

**STRUCTURAL REQUIREMENTS OF ORGANIC ANION TRANSPORTING
POLYPEPTIDE MEDIATED TRANSPORT**

by

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of the requirements for the degree of Doctor of Philosophy.

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Dedication

I would like to dedicate the work herein to my parents and my grandparents.

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I also want to thank Ben's family: Mom, Dad, Julie, Brian, Katharine, James, Jeff, Ryan, Savannah, Butterfly and Jacy. You are my family and thank you for your love and prayers for Ben and me.

Last but not least, to my husband Ben: Thank you, love. You are the light in my darkness. I love you.

ABSTRACT

The organic anion transporting polypeptides (human: OATP; other: Oatp) form a mammalian transporter superfamily that mediates the transport of structurally unrelated compounds across the cell membrane. Members in this superfamily participate in the absorption, distribution and excretion of many endogenous and exogenous substances including a number of medications and environmental toxicants. Polymorphisms of OATPs have been shown clinically to give rise to inter-individual variabilities of drug efficacy and/or toxicity. Furthermore, as multi-specific transporters, they are potential sites for drug-drug interactions. Therefore, understanding the mechanism of OATP/Oatp mediated transport of endo- and xenobiotics will not only help to improve drug efficacy but also to improve the prediction and prevention of toxicity. The overall goal of this dissertation is identifying key amino acids that may play an important role in OATP/Oatp-mediated transport and investigating the spatial size of the substrate binding/translocation pocket. In this dissertation, I defended three specific aims. In the first specific aim, I evaluated the hypothesis that conserved positively charged amino acids play important roles in OATP1B1 transport function. To address this aim, site-directed mutagenesis was employed and the mutants of several conserved positively charged amino acids were studied. The two extracellular amino acids R57 and K361 were found to be important in OATP1B1 mediated transport of estradiol-17 β -glucuronide, estrone-3-sulfate and BSP. In the second specific aim, I evaluated the hypothesis that quantifying transport activities of different substrates mediated by chimeras between rat Oatp1a1 and

Oatp1a4 in combination with site-directed mutagenesis should allow us to identify regions and/or individual amino acids that are important for Oatp1a4-mediated substrate recognition and/or transport. The effects of chimeric proteins on transport activity were substrate dependent. Extracellular loop 4 and transmembrane domain 8 were identified to be important in transport of digoxin, taurocholate and estradiol-17 β -glucuronide. The C-terminal half of Oatp1a4 and Oatp1a1 was found to be important for BSP transport and the interactions between the N-terminal half and the C-terminal half of Oatp1a4 is essential for DPDPE transport. In the third specific aim, I evaluated the hypothesis that different rat renal organic anion transporters of the Oat and Oatp families selectively transport perfluorinated carboxylates (PFCAs) depending on the chain lengths. The purpose of this study was to determine the substrate size selectivity of Oats and Oatp1a1. To address this aim, the inhibitory effects of PFCAs with chain length from C2 to C18 on transport of model substrates by rat Oat1, Oat2, Oat3, Urat1 and Oatp1a1 was quantified. Furthermore, direct uptake of the best inhibitors was characterized. The best substrates for Oats were C7 and C8, whereas Oatp1a1 transported longer PFCAs such as C9 and C10 better than C8 and C7. Altogether, this dissertation reveals that (1) some conserved positively charged amino acids and the C-terminal half are important for OATP/Oatp mediated transport of certain substrates; (2) OATP/Oatps have several binding/translocation sites for different substrates and (3) the preferred substrate size for OATP/Oatps is slightly bigger than that for Oats.

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List of Abbreviations

ABC	= ATP-binding cassette
AhR	= aryl hydrocarbon receptor
ASBT	= apical sodium bile acid transporter
BSP	= bromosulfophthalein
C10	= perfluorodecanoate
C11	= perflouroundecanoate
C4	= perfluorobutyrate
C6	= perfluorohexanoate
C7	= perfluoroheptanoate
C8	= perfluorooctanoate
C9	= perfluorononanoate
CAR	= constitutive androstane receptor
CCK8	= cholecystokinin 8
CHO	= Chinese hamster ovary
CNS	= central nervous system
CoMFA	= comparative molecular field analysis
CYP450	= cytochrome P-450
DMEM	= Dulbecco's Modified Eagle Medium
DPDPE	= D-penicillamine^{2,5}-enkephalin

ECL	= enhanced chemiluminescence
EL	= extracellular loop
GLUT	= glucose transporters
HEK293	= human embryonic kidney cells clone 293
HPLC	= High performance liquid chromatography
IL-6	= interleukin-6
kDa	= kilodaltons
K_m	= Michaelis constant
LC	= liquid chromatography
LPS	= lipopolysaccharide
MDR	= multidrug resistance
MFS	= major facilitator superfamily
mg	= milligram
min	= minute
MS	= mass spectrometry
MTS	= methanethiosulfonate
NF-κB	= nuclear factor κ-light-chain-enhancer of activated B cells
Nrf2	= nuclear factor erythroid 2-related factor 2
OAT	= organic anion transporters
OATP/Oatp	= organic anion transporting polypeptides

ORF	=	open reading frame
PAH	=	<i>p</i>-aminohippurate
PBS	=	phosphate buffered saline
PCR	=	polymerase chain reaction
PFCA	=	perfluorinated carboxylates
PFOA	=	perfluorooctanoate
pmol	=	picomole
PNGase F	=	N-Glycosidase F
PPAR-α	=	peroxisome proliferator-activated receptor α
PXR	=	pregnane X receptor
QSAR	=	quantitative structure-activity relationship
SLC	=	solute carrier
SNP	=	single nucleotide polymorphisms
TLR4	=	toll-like receptor 4
TM	=	transmembrane
TNF-α	=	tumor necrosis factor-α
V_{max}	=	maximum rate
WT	=	wild type

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

I. INTRODUCTION TO MEMBRANE TRANSPORTERS

Membrane transporters are proteins with the ability to move molecules across biological membrane borders in living cells. They control both intercellular and intracellular exchange of nutrients, ions, metabolites, signaling molecules and xenobiotics. The essential physiological roles of transporters are supported by the fact that transporters are ubiquitously present in bacteria, archaea and eukarya (Saier, 2000).

Organic anion transporting polypeptides (human OATPs; rodent Oatps) form a superfamily of membrane transporters that mediate the movement of a wide range of amphipathic organic compounds across plasma membranes (Hagenbuch and Meier, 2003; Hagenbuch and Meier, 2004). Together with organic anion transporters (OATs), they control most organic anion uptake in all mammalian organisms. The aim of the studies presented in this dissertation is to understand the structural requirements of the transporter and the structural requirements of the substrates for the transport activities of OATP/Oatps in the context of their physiological, pharmacological and toxicological roles.

In this dissertation, I will first introduce the classification systems and related nomenclatures of transporters that will be helpful later in understanding both the published studies and the primary research reported in this dissertation.

Then I will provide some details regarding the OATP/Oatp superfamily focusing on their biological and toxicological roles as well as on structure-function characterizations. From the review of published literature, I will build the framework required for designing the primary research reported in subsequent chapters. In conjunction with other published results, these data begin to reveal some critical structural features of both transporters and substrates required for OATP/Oatp mediated transport.

II. CLASSIFICATION OF TRANSPORTERS

Due to the various transport mechanisms and diversified substrate spectra, there are multiple classification systems for membrane transporters. Based on mechanisms and energy source, membrane transporters can be divided into 2 broad classes: passive transporters and active transporters. Passive transporters include ion channels (such as Na^+ channels) and facilitated diffusion transporters (such as the glucose transporters of the GLUT family). Active transporters can be further divided into primary active and secondary active transporters. Primary active transporters such as the Na^+/K^+ ATPase directly use ATP as energy source. Secondary active transporters, such as the Na^+ / amino acid symporters and the Na^+/H^+ exchanger, use ion gradients established, for example, by the Na^+/K^+ ATPase as their energy source (Saier, 2000).

The two largest superfamilies of membrane transporters are the ATP-binding cassette (ABC) superfamily and the major facilitator superfamily (MFS).

ABC transporters generally have multiple transmembrane components, capable of transporting both small molecules and macromolecules in response to ATP hydrolysis. Members of the ABC superfamily are primary active transporters such as the human ABCB1 (MDR1) (Jones and George, 2004). The MFS transporters are single-polypeptide carriers capable of transporting small solutes. Members of the MFS superfamily can be either active transporters, such as the glycerol-3-phosphate transporter, or passive transporters, such as GLUT2 (Pao et al., 1998).

All genes encoding mammalian transporters either belong to *SLC* (Solute Carrier) families or non-*SLC* families based on substrate specificity and amino acid sequence identities. A transporter belongs to a specific *SLC* family if it has at least 20–25% amino acid sequence identity to existing members in that family (Hediger et al., 2004). *SLC* families include genes encoding passive transporters, ion-coupled transporters and exchangers for different kinds of substrates. A list of current *SLC* families is shown in Table 1-1. Examples of *SLC* transporters include glucose transporters, metal transporters, and organic anion transporters. Examples of non-*SLC* transporters include ABC transporters, ion channels, and water channels.

Table 1-1: The HGNC solute carrier family series

<i>SLC1</i> : The high affinity glutamate and neutral amino acid transporter family
<i>SLC2</i> : The facilitative GLUT transporter family
<i>SLC3</i> : The heavy subunits of the heteromeric amino acid transporters
<i>SLC4</i> : The bicarbonate transporter family
<i>SLC5</i> : The sodium glucose cotransporter family
<i>SLC6</i> : The sodium- and chloride- dependent neurotransmitter transporter family
<i>SLC7</i> : The cationic amino acid transporter/glycoprotein-associated family
<i>SLC8</i> : The Na ⁺ /Ca ²⁺ exchanger family
<i>SLC9</i> : The Na ⁺ /H ⁺ exchanger family
<i>SLC10</i> : The sodium bile salt cotransport family
<i>SLC11</i> : The proton coupled metal ion transporter family
<i>SLC12</i> : The electroneutral cation-Cl cotransporter family
<i>SLC13</i> : The human Na ⁺ -sulfate/carboxylate cotransporter family
<i>SLC14</i> : The urea transporter family
<i>SLC15</i> : The proton oligopeptide cotransporter family
<i>SLC16</i> : The monocarboxylate transporter family
<i>SLC17</i> : The vesicular glutamate transporter family
<i>SLC18</i> : The vesicular amine transporter family
<i>SLC19</i> : The folate/thiamine transporter family
<i>SLC20</i> : The type III Na ⁺ -phosphate cotransporter family
<i>SLCO</i> : The organic anion transporting family
<i>SLC22</i> : The organic cation/anion/zwitterion transporter family
<i>SLC23</i> : The Na ⁺ -dependent ascorbic acid transporter family
<i>SLC24</i> : The Na ⁺ /(Ca ²⁺ -K ⁺) exchanger family
<i>SLC25</i> : The mitochondrial carrier family
<i>SLC26</i> : The multifunctional anion exchanger family
<i>SLC27</i> : The fatty acid transport protein family
<i>SLC28</i> : The Na ⁺ -coupled nucleoside transport family
<i>SLC29</i> : The facilitative nucleoside transporter family
<i>SLC30</i> : The zinc efflux family
<i>SLC31</i> : The copper transporter family
<i>SLC32</i> : The vesicular inhibitory amino acid transporter family
<i>SLC33</i> : The Acety-CoA transporter family
<i>SLC34</i> : The type II Na ⁺ -phosphate cotransporter family
<i>SLC35</i> : The nucleoside-sugar transporter family
<i>SLC36</i> : The proton-coupled amino acid transporter family
<i>SLC37</i> : The sugar-phosphate/phosphate exchanger family
<i>SLC38</i> : Family of System A and System N sodium-coupled neutral amino acid transporters
<i>SLC39</i> : The metal ion transporter family
<i>SLC40</i> : The basolateral iron transporter family
<i>SLC41</i> : The MgtE-like magnesium transporter family
<i>SLC42</i> : The Rh ammonium transporter family
<i>SLC43</i> : Na ⁺ -independent, system-L like amino acid transporter family
<i>SLC44</i> : Choline-like transporter family
<i>SLC45</i> : Putative sugar transporter family
<i>SLC46</i> : Folate transporter family
<i>SLC47</i> : Multidrug and Toxin Extrusion (MATE) family
<i>SLC48</i> : Heme transporter family

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III. INTRODUCTION TO OATPS

A. Basic information and history

Organic anion transporting polypeptides (human: OATPs; all other species: Oatps; gene symbol: *SLCO/Slco*; Old gene symbol: *SLC21A/Slc21a*) form a superfamily within the *SLC* families that mediate the sodium-independent transmembrane transport of a wide range of amphipathic endogenous and exogenous organic compounds (Hagenbuch and Meier, 2003; Hagenbuch and Meier, 2004). The first Oatp was cloned and characterized from rat liver (Oatp1a1) in 1994 (Jacquemin et al., 1994). In 2004, a new nomenclature for this superfamily of transporters was introduced (Hagenbuch and Meier, 2004). The entire superfamily is classified into 6 families (families OATP1/Oatp1 through OATP6/Oatp6) and each family, into subfamilies (*e.g.*, OATP1A, OATP2B). Each individual family contains proteins with amino acid sequence identities of more than 40%; subfamilies contain proteins with amino acid sequence identities of more than 60%. Individual paralogues within a subfamily are indicated by different final numbers (*e.g.*, Oatp1a1, OATP1A2, Oatp1a3, Oatp1a4, *etc.*). More than 160 OATP/Oatps have been predicted in at least 25 different species. However, only a few OATP/Oatps have been functionally characterized (Hagenbuch and Gui, 2008).

In mammals, many of the OATP/Oatp transporters are distributed in multiple cell types or organs, whereas some of them are predominantly expressed in one or two cell types or organs. For example, OATP1B1 and OATP1B3 are

considered to be exclusively expressed in human hepatocytes; OATP1A2 has strongest expression in brain, whereas it is also expressed in multiple organs such as kidney, liver, ciliary body and intestine. OATP/Oatps exhibit broad and overlapping substrate specificity. The cell type or organ distribution and model substrates of most of the common OATP/Oatps are summarized in several reviews (Hagenbuch and Meier, 2003; Hagenbuch and Meier, 2004; Konig et al., 2006; Hagenbuch and Gui, 2008). Table 1-2 is a list of basic information about the OATP1/Oatp1 family, and the primary research presented in later chapters will focus on several members in this family.

B. OATPs and human health

The roles of OATPs in human health have been extensively studied in various organs such as brain, liver and kidney for the past ten years. These studies have addressed the roles of OATPs in the context of physiology, pharmacology and toxicology.

(1) Distribution of OATPs in normal human organs

Three OATPs have been shown to be expressed in the brain. OATP1A2 is expressed in the endothelial cells of the blood brain barrier (Kullak-Ublick et al., 1995; Gao et al., 2000). Given the location and broad substrate spectrum of OATP1A2, it may be involved in delivering CNS drugs or drugs with CNS side effects across the blood-brain barrier. A number of drugs or toxins that exhibit either CNS efficacy or toxicity have been shown to be OATP1A2 substrates, such

Table 1-2: Members in OATP1/Oatp1 family

Gene symbol	Protein name	Other names	Species	Predominant substrate	Organ distribution
<i>Slco1a1</i>	Oatp1a1	Oatp1	Rodent	Bile salts, organic anions, organic cations	Liver, kidney, choroid plexus
<i>SLCO1A2</i>	OATP1A2	OATP-A	Human	Bile salts, organic anions, organic cations	Brain, kidney, liver, ciliary body
<i>Slco1a3</i>	Oatp1a3_v1 Oatp1a3_v2	OAT-K1 OAT-K2	Rodent	Bile salts, organic anions	Kidney
<i>Slco1a4</i>	Oatp1a4	Oatp2	Rodent	Digoxin, bile salts, organic anions	Liver, Brain, choroid plexus, ciliary body, retina
<i>Slco1a5</i>	Oatp1a5	Oatp3	Rodent	Bile salts, organic anions	Jejunum, choroid plexus
<i>Slco1a6</i>	Oatp1a6	Oatp5	Rodent		
<i>SLCO1B1</i>	OATP1B1	OATP-C, LST-1, OATP2	Human	Bile salts, organic anions	Liver
<i>Slco1b2</i>	Oatp1b2	Oatp4, Lst-1	Rodent	Bile salts, organic anions	Liver, ciliary body
<i>SLCO1B3</i>	OATP1B3	OATP8	Human	Bile salts, organic anions, digoxin	
<i>SLCO1C1</i>	OATP1C1	OATP-F OATP-RP5	Human	T3, rT3, BSP	Brain, Leydig cells
<i>Slco1c1</i>	Oatp1c1	Oatp14, BSAT1	Rodent		

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as D-penicillamine^{2,5}-enkephalin (DPDPE) (Gao et al., 2000), methotrexate (Badagnani et al., 2006), levofloxacin (Maeda et al., 2007) and microsynin (Fischer et al., 2005). OATP1C1, a high-affinity thyroxine transporter, is also located in the brain (Pizzagalli et al., 2002). OATP1C1 has been suggested to be essential for thyroid hormone delivery to the developing brain (Hagenbuch, 2007). In addition, a number of nonsteroidal antiinflammatory drugs in the fenamate class have been shown to competitively inhibit thyroxine transport mediated by OATP1C1 (Westholm et al., 2009). Taken together, OATP1C1 thought to be an important thyroid hormone transporter and a potential drug-drug interaction site in the brain. OATP2B1 is expressed at the luminal membrane of brain endothelial cells (Bronger et al., 2005). OATP2B1 has relatively narrow substrate specificity at pH 7.4. OATP2B1 transports bromosulfophthalein (BSP) (Kullak-Ublick et al., 2001), estrone-3-sulfate and dehydroepiandrosterone-3-sulfate (Pizzagalli et al., 2003). However, at lower pH, additional substrates have been reported such as taurocholate and statins (Nozawa et al., 2004). Therefore, the pH of the micro environment might be important for OATP2B1 transport activity.

Several OATPs are expressed in liver, the primary detoxification organ. OATP1B1, a liver-specific OATP (Abe et al., 1999; Hsiang et al., 1999; Konig et al., 2000), is localized to the basolateral membrane of hepatocytes (Konig et al., 2000). In addition to transporting endogenous metabolites such as bilirubin (Briz et al., 2003) and taurocholate (Abe et al., 1999; Hsiang et al., 1999; Kullak-Ublick et al., 2001), OATP1B1 also mediates the liver uptake of a large number of drugs

and toxins (Hagenbuch and Gui, 2008). The potential drug-drug interactions via OATP1B1 and the impact of OATP1B1 polymorphisms on the pharmacokinetics of several drugs such as rifampicin (Vavricka et al., 2002; Tirona et al., 2003; Treiber et al., 2007; van Giersbergen et al., 2007), pravastatin, rosuvastatin, pitavastatin (Nishizato et al., 2003; Morimoto et al., 2004; Mwinyi et al., 2004; Niemi et al., 2004; Simonson et al., 2004; Tachibana-Iimori et al., 2004; Chung et al., 2005; Kameyama et al., 2005; Hirano et al., 2006; Maeda et al., 2006; Noe et al., 2007; Seithel et al., 2007; Kitamura et al., 2008; Neuvonen et al., 2008; Vaidyanathan et al., 2008; Furihata et al., 2009; Qiang et al., 2009) and irinotecan (SN-38) (Nozawa et al., 2005; Rohrbacher et al., 2006) have been extensively studied in both cell lines and clinical settings.

Another liver-specific OATP is OATP1B3, which has 80% amino acid identity to OATP1B1 (König et al., 2000; Abe et al., 2001). In addition to many substrates shared with OATP1B1, OATP1B3 also has its own specific substrates, such as cholecystokinin octapeptide (CCK-8) and the anticancer drugs paclitaxel and docetaxel (Ismair et al., 2001; Kullak-Ublick et al., 2001; Smith et al., 2005). Oatp1b2 is the rodent orthologue of OATP1B1 and OATP1B3. In 2008, Oatp1b2 knockout mice were reported (Lu et al., 2008; Zaher et al., 2008). These mice showed no morphological abnormalities. They were used to further characterize the role of Oatp1b2 in the pharmacokinetics of pravastatin and rifampin (Zaher et al., 2008) and in the hepatic uptake/toxicity of phalloidin and microcystin-LR (Lu et al., 2008) *in vivo*. In addition to the two liver-specific OATPs, several other

OATPs including OATP1A2, OATP2A1, OATP2B1 and OATP4A1 (Hagenbuch and Gui, 2008) are also expressed in the liver. Except for OATP2A1, which may be involved in the regulation of pericellular prostaglandin levels (Schuster, 2002) and OATP1A2 which has been localized to cholangiocytes (Lee et al., 2005), all the other OATPs in the liver are thought to play important roles in the absorption and disposition of numerous endobiotics and xenobiotics (Hagenbuch and Gui, 2008).

In contrast to liver, there are only two major OATPs expressed in the kidney: OATP4C1 and OATP1A2. OATP4C1 is localized to the basolateral membrane of the proximal tubule (Mikkaichi et al., 2004) whereas OATP1A2 is localized to the brush border membrane in the distal nephron (Lee et al., 2005). OATP4C1 is considered a kidney-specific transporter. In addition to its possible function of delivering thyroid hormone, OATP4C1 also transports a number of drugs such as digoxin, ouabain and methotrexate into kidney, and thus may play an important role in the renal elimination and disposition of these medications. OATP1A2 is expressed in multiple organs, including brain (Gao et al., 2000), kidney (Lee et al., 2005) and intestine (Glaeser et al., 2007). Therefore, OATP1A2 is likely involved in the disposition of a number of drugs and potentially serves as a drug-drug and drug-food interaction site. A good example of this phenomenon is OATP1A2 mediated uptake of fexofenadine, and its interactions with other xenobiotics (Cvetkovic et al., 1999; Dresser et al., 2002; Dresser and Bailey,

2003; Dresser et al., 2005; Kamath et al., 2005; Shimizu et al., 2005; Bressler, 2006; Kikuchi et al., 2006; Glaeser et al., 2007; Matsushima et al., 2008; Greenblatt, 2009; Qiang et al., 2009).

(2) Expression of OATPs in cancer

In addition to the normal expression of OATPs in various organs, several types of cancers have recently been shown to overexpress certain OATPs. For example, the liver-specific OATP1B3 has been detected in gastrointestinal cancers (Abe et al., 2001; Lee et al., 2008), breast cancer (Muto et al., 2007; Wlcek et al., 2008) and prostate cancer (Hamada et al., 2008). Another liver-specific transporter, OATP1B1 has been found in neoplastic colon tissue (Ballesterio et al., 2006). In addition, OATP1A2 mRNA levels have been shown to be high in breast cancer, compared to the adjacent healthy tissue (Meyer zu Schwabedissen et al., 2008). Because several OATPs, such as OATP1B1, OATP1B3 and OATP1A2 are able to transport hormones and hormone conjugates (Hagenbuch and Gui, 2008), the expression of OATPs may contribute to hormone-dependent growth of cancerous cells, and anti-apoptotic progression of cancer (Muto et al., 2007; Hamada et al., 2008; Lee et al., 2008; Meyer zu Schwabedissen et al., 2008). However, OATPs are also able to transport anti-cancer drugs such as methotrexate and paclitaxel (Hagenbuch and Gui, 2008). Therefore, OATPs are candidate carriers that could deliver anti-cancer drugs to cancer cells. The studies of the function and expression of OATPs under pathological conditions are undoubtedly very valuable to understand and treat cancers.

(3) OATP/Oatp expression in response to endogenous compounds and xenobiotics

Hepatocyte nuclear factor 1 alpha has been shown to regulate the liver-specific expression of OATP1B1 and OATP1B3 (Jung et al., 2001). The expression of OATP/Oatps is also influenced by endogenous and exogenous stimulants. It has been shown that in rodents, sex hormones regulate the sex specific expression of Oatps in kidney (Lu et al., 1996; Cheng and Klaassen, 2006; Cheng and Klaassen, 2009) and liver (Rost et al., 2005; Cheng and Klaassen, 2006; Cheng et al., 2006). Prolactin and growth hormone can upregulate rat Oatp1b2, and human OATP1B3 gene expression via the Stat5 signal-transduction pathway (Wood et al., 2005). Growth hormone can also regulate Oatp1a1 expression in mouse liver but not in kidney (Cheng et al., 2006). The proinflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) (Vee et al., 2009), interferon-gamma (Le Vee et al., 2010), and hepatocyte growth factor (Le Vee et al., 2009) down-regulate the expression of several OATPs in human hepatocytes. However, using knock-out mouse models, the down-regulation of Oatp1a1 after lipopolysaccharide (LPS) or bile-duct ligation induced cholestasis has been shown to be independent of the individual activity of TNF- α , IL-1, IL-6, or NF- κ B (Lickteig et al., 2007). These findings suggest that physiologically and pathologically, there is more than one pathway involved in the regulation of OATP/Oatp expression at the transcriptional level.

In addition to endogenous regulators, a number of xenobiotics can also affect the expression of OATP/Oatps. It has been shown in mouse, rats, and humans that inducers of the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor α (PPAR- α), and nuclear factor erythroid 2-related factor 2 (Nrf2) can modulate the expression of OATP/Oatps in either liver, kidney or intestine (Guo et al., 2002; Cheng et al., 2005; Cheng et al., 2006; Jigorel et al., 2006). In addition, LPS can down-regulate Oatp1b2 in mouse liver through the toll-like receptor 4 (TLR4) (Li et al., 2004). Moreover, perfluorooctanoic acid and perfluorodecanoic acid can down-regulate Oatps in mouse liver through PPAR- α pathways (Cheng and Klaassen, 2008). Kidney Oatps are differentially regulated after cisplatin treatment (Aleksunes et al., 2008).

Compared to the transcriptional regulation mediated by endogenous molecules, the body has additional systems to regulate OATP/Oatp expression in response to exposure to xenobiotics. For a multispecific transporter superfamily that can transport many classes of compounds, including nutrients and toxicants, a fine-tuned regulating system is essential for adaptation to the changing environment.

In summary, as a superfamily of multispecific transporters, OATPs are able to deliver different classes of substrates, both endogenous and exogenous, to different parts of the body. They play important roles in 1) maintaining the normal

physiological metabolite flow in the body, 2) delivering drugs and toxicants to the site of action, and 3) defending the body from environmental and biological toxins. Furthermore, the abnormal expression of OATPs in cancers may contribute to the pathogenesis of cancers, but may also represent a potential mechanism for the uptake of anti-cancer drugs. The importance of OATP/Oatps, both physiologically and/or pathologically, has inspired many researchers to study this superfamily of transporters at the molecular level. In the next section, I will review studies performed by several groups analyzing the structure and function relationships of OATP/Oatps using different approaches.

C. Structural features of OATP/Oatps

As a superfamily of membrane proteins, there are several common structural features that all OATP/Oatps share. First, membrane protein topology programs suggest that all OATP/Oatps contain 12-transmembrane (TM) domains, with both N- and C- termini inside the cell. This 12-TM topology has been verified experimentally for rat Oatp1a1 by immunofluorescence analysis, using an epitope-specific antibody designed to differentiate a 10- from a 12-transmembrane domain model (Wang et al., 2008). Second, all OATP/Oatps have a large extracellular loop between TMs 9 and 10 that contains many conserved cysteine residues. Site-directed mutagenesis of these conserved cysteines has been performed for OATP2B1. The results suggest that all 10 extracellular cysteines normally form disulfide-bonds that are important in the trafficking and function of OATP2B1 (Hanggi et al., 2006). Third, there are N-glycosylation sites in

extracellular loops 2 and 5 (Lee et al., 2003). The glycosylation sites of Oatp1a1 have been mapped to asparagines 124 (extracellular loop 2), 135 (extracellular loop 2), and 492 (extracellular loop 5) (Lee et al., 2003; Wang et al., 2008). Finally, all OATP/Oatps share the OATP “superfamily signature”: a stretch of highly conserved consensus amino acids D-X-RW-(I,V)-GAWW-XG-(F,L)-L at the border of extracellular loop 3 and TM6 (Hagenbuch and Meier, 2003).

Several OATP/Oatps, including OATP1A2, OATP2B1 and rat Oatp1a1, have a PDZ consensus binding site at their C termini (Wang et al., 2005). Oligomerization of Oatp1a1 with PDZK1, via this consensus sequence, has been shown using cell lines and PDZK1 knock out mice to be very important for proper cell surface localization and function of Oatp1a1 (Wang et al., 2005). PDZK1 is a major scaffold protein and is important in determining the location of many membrane-associated proteins to defined areas of the plasma membrane (Gisler et al., 2003; Kocher et al., 2003). The presence of PDZ consensus binding sites in several OATP/Oatps indicates that PDZK1 may play a role in the subcellular localization of some OATP/Oatps.

Figure 1-1 summarizes the general features of OATP/Oatps. However, more information is needed to answer the question: how do these transporters carry substrates across the membrane?

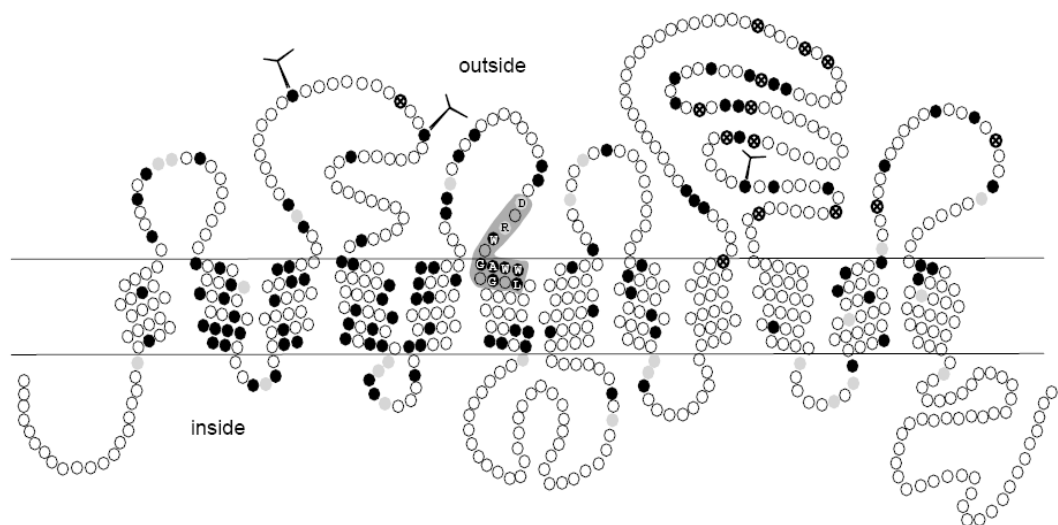


Figure 1-1 General features of OATP/Oatps. OATP/Oatp proteins are predicted to have 12-transmembrane domains with both N- and C- termini inside the cell. Highly conserved amino acids among most OATP/Oatps are indicated in black. Conserved and charged amino acids (D, E, K, R) are given in gray, and conserved cysteines (C) are marked with asterisks. Three potential N-glycosylation sites (Y) predicted for rat Oatp1a1 are present on extracellular protein loops. The OATP superfamily signature is indicated at the border of the extracellular loop 3 and the transmembrane domain 6.

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Scientists have evaluated the transport process using two major approaches. One is to investigate how the transporter interacts with its substrates. In other words, which parts of the transporter are important for substrate binding, and what structural requirements are needed for the transporter to complete the translocation process? The other approach is to concentrate on the substrates. One can determine what structural requirements are needed for a molecule to be a substrate, and what other molecules are required for the movement of the substrate. In the field of OATP/Oatps, researchers have studied the transport mechanisms from both perspectives during the past 15 years. The approaches and findings are discussed below in detail.

D. Structure and function studies of OATP/Oatps

(1) Structural requirement of OATP/Oatps for proper transport function.

(i) Crystallography

X-ray crystallography is the most direct way to characterize transporter protein structures and to study transport mechanisms, especially if the protein can be crystallized in the presence of substrates. However, because of the difficulties associated with expression and crystallization of membrane proteins, the crystal structures of very few membrane proteins are available. In the major facilitator superfamily, only three crystal structures have been solved for 12 TM membrane transporters: lactose permease of *Escherichia coli* (*E. coli*) (Abramson et al., 2003), glycerol-3-phosphate transporter of *E. coli* (Huang et al., 2003) and the multidrug transporter EmrD of *E. coli* (Yin et al., 2006). Despite the low sequence

similarity of these three membrane transporters, the reported structures are highly similar with 12-transmembrane helices. This led to the hypothesis that all members of the MFS share a similar structure, regardless of their low sequence identity (Vardy et al., 2004). The importance of this structural homology will be considered in the following discussion on computer modeling and mutagenesis.

(ii) Computer modeling

Computational or *in silico* methods have been adopted widely to study structure-function relationship of transporters, especially in the absence of crystal structures. There are two main approaches to modeling the structure-function relationship of transporters: transporter-based modeling and substrate-based modeling. I will introduce the transporter-based methods in this section and the substrate-based methods will be discussed in later sections. In transporter-based modeling, at least one crystal structure of a template protein is required. This template must have a high level of homology with the target transporter. The homology requirement can be either sequence homology, such as high amino acid sequence identities, or structural homology, such as the same number of transmembrane domains. After selecting the best fit template, the next step is sequence alignment of the target protein with the template. Based on the alignment, a 3D structure model of the target protein is then generated, optimized, and validated (Chang and Swaan, 2006). Furthermore, using the 3D model of the target protein, important amino acids can be predicted, and docking studies can be performed. A docking study describes the “best-fit” orientation of a substrate that

binds to the target transporter model, and predicts the putative binding or substrate-protein interaction domains. In recent years, three dimensional models have been generated for more than 20 membrane transporters, such as the human apical sodium bile acid transporter (ASBT) (Zhang et al., 2002; Zhang et al., 2004), the human organic anion transporter 1 (Oat1) (Perry et al., 2006) and human OATP1B3 and OATP2B1 (Meier-Abt et al., 2005). The great advantage of generating a computer model is that the model may provide information regarding the orientation and spatial position of amino acids that may be important in structure or function of the transporter. In other words, a computer model is a very powerful hypothesis generator for subsequent studies, such as site-directed mutagenesis.

(iii) Polymorphisms studies

Naturally occurring polymorphisms have provided some insights into the structure-function relationship for OATP/Oatps. Polymorphisms in OATP/Oatps encode the same transporter with one or a few mutated amino acids. The most common polymorphism is the single nucleotide polymorphisms (SNPs), which results in a point mutation in the primary structure of the transporter. Sometimes, a combination of two or more SNPs can also be found in certain populations. Studying the transport function of different polymorphisms allows evaluating the importance of a particular amino acid for the structure and/or function of the transporter. Because OATP1A2, OATP1B1, and OATP1B3 are highly involved in

drug disposition, the effects of polymorphisms on transport activities of these two transporters have been examined.

For OATP1A2, E172D (A516C), N135I (A404T), and T668S (C2003G) variants have altered expression of the transporter in the plasma membrane, which may contribute to the reduced transport activity (Lee et al., 2005; Badagnani et al., 2006). The N135I variant has also been shown to have alterations in glycosylation status (Lee et al., 2005).

At least 15 alleles have been identified for OATP1B1 polymorphisms (Fahrmayr et al., 2010). OATP1B1*5 (V174A, T521C) and OATP1B1*15 (V174A, T521C + N130D, A388G) influence the pharmacokinetics or pharmacodynamics of drug substrates the most. Extensive clinical research has been done to study the effects of these two polymorphisms on efficacy, clearance, and toxicity of a number of drugs including statins, rifampicin, and irinotecan (Fahrmayr et al., 2010). In HEK293 and HeLa cell expression systems, OATP1B1*5 and OATP1B1*15 have been shown to have decreased transport activities. This reduced transport rate may be due to decreased protein expression on the cell surface (Kameyama et al., 2005) or due to impaired translocation processes (Iwai et al., 2004).

Compared to OATP1B1, only a few known polymorphisms are known for OATP1B3. S112A (T334G) and M233I (G699A) are the two best characterized

polymorphisms. In stably transfected MDCK II cells, the transport rate of several OATP1B3 model substrates is slightly higher in S112A and M233I mutants than in wild-type OATP1B3. However, in HEK293 cells, the transport rate is the same between wild-type OATP1B3 and these two mutants (Letschert et al., 2004). Clinical studies have shown that patients with S112A and M223I polymorphisms have higher serum concentrations of digoxin (Miura et al., 2007) and enhanced enterohepatic circulation of mycophenolic acid (Tsujimoto et al., 2008). However, the clearance of both unbound and total paclitaxel was not significantly associated with the S112A or M223I polymorphisms (Smith et al., 2007).

(iv) Chimeric proteins

Another strategy to study structure-function relationships is using chimeric proteins. This strategy works best when there are at least two or more orthologs or paralogs that share high amino acid sequence identities but different substrate spectra. In the case of OATP/Oatps, members in the same subfamily such as human paralogs OATP1B1 and OATP1B3, rat paralogs Oatp1a1 and Oatp1a4, or even human OATP1A2 and its rat ortholog Oatp1a1 are all very good candidate pairs for building chimeras. The aim of constructing chimeric proteins is to isolate the essential structure or amino acid sequence that makes one OATP/Oatp different from the other. Although OATP/Oatps in the same subfamily share very similar primary, secondary and even tertiary structures, there is much evidence that they are able to transport distinct substrates (Hagenbuch and Meier, 2003; König et al., 2006; Hagenbuch and Gui, 2008). Gui and Hagenbuch (2008)

constructed chimeric proteins between OATP1B1 and OATP1B3. They replaced single transmembrane domains of OATP1B3 with corresponding domains from OATP1B1 one at a time. Using this technique, they have shown that TM10 in OATP1B3 plays an essential role in recognition and/or translocation of CCK-8, an OATP1B3 specific substrate (Gui and Hagenbuch, 2008). In addition, the same strategy has been applied using OATP1B1 as the backbone, and TM8, TM9 (Miyagawa et al., 2009), and TM10 (Gui and Hagenbuch, 2009) of OATP1B1 have been shown to affect the transport of estrone-3-sulfate and/or estradiol-17 β -glucuronide.

(v) Site-directed mutagenesis

In order to identify the roles of individual amino acid residues involved in transport, site-directed mutagenesis has been a useful approach. By changing a target amino acid to another amino acid, a mutant protein is generated with identical or slightly different structure. When combined with functional assays, this method provides useful information about the role of a particular amino acid in the transport of a substrate. In principle, most point mutations do not dramatically alter function. However, transport function will be altered or destroyed when some critical amino acids are mutated. This may be due to either disrupting the interaction of the substrate to the transporter by the mutated amino acid, or due to altered conformational changes that are normally required for translocation of the substrates. In addition, loss of function may also occur due to misfolding of the protein or a lack of surface expression. A successful example

using this technique is the structure-function relationship studies of lactose permease performed prior to the report of its crystal structure. Several key amino acids were identified using site-directed mutagenesis, and later, they were confirmed by crystal structure, to be critical in substrate binding and translocation (Frillingos et al., 1998; Kaback et al., 2001; Abramson et al., 2003). Mutagenesis is currently widely used in structure and function relationship studies of membrane transporters for which the crystal structure is not available. The most common strategy is to replace a residue with alanine. Alanine has a non-polar aliphatic side chain with relatively small steric effects. In site-directed mutagenesis, it has been widely used as a substitute for amino acids with charged, aromatic, or polar side chains. Using the alanine side chain to replace a functional group on other amino acids not only reduces the chance of introducing additional steric hindrance, but more importantly, reveals the potential effects of the replaced functional group on the structure and/or function of the transporter. Site-directed mutagenesis, especially alanine mutations, have been used in studying the structure-function relationship of many transporters *e.g.* hOAT1 (human organic anion transporter 1 *SLC22A6*). Using this technique, amino acids in TM1 (Hong et al., 2004), TM7 (Hong et al., 2007), the C-terminus (Xu et al., 2006) as well as several aromatic amino acids (Perry et al., 2006) have been shown to be important either for transport function and/or structural stability.

(vi) Cysteine scanning mutagenesis

Cysteine scanning mutagenesis is another widely used strategy in which consecutive residues are changed to cysteines. Chemical modifications of the introduced cysteine residues can provide information about the accessibility of a particular residue to the extracellular space. The purpose of the cysteine scanning is to locate the boundaries of the transmembrane domain and the interface of substrates and the transporter. This technique was first introduced by Arthur Karlin and Myles Akabas at Columbia University to identify channel-lining residues of the nicotinic acetylcholine receptor (nAChR) (Karlin and Akabas, 1998). Shortly after its development, this method was widely used in many areas, including examination of channel domains, mapping of ligand-gated channel binding sites, mapping of receptor binding sites and transporter binding sites (Newell and Czajkowski, 2007). In principle, the backbone of the “wild-type” transporter should contain no reactive thiol (-SH) groups. Cysteine residues are then introduced into the wild-type protein by site-directed mutagenesis to replace the amino acid residues to be probed. Subsequently, -SH-specific reagents, such as derivatives of methanethiosulfonate (MTS) are applied to probe the physico-chemical nature of the environment. MTS reagents can react with cysteine residues and only label the free -SH groups that are exposed to the surrounding environment. Depending on different sizes, the smaller the MTS reagent is, the deeper inside the protein it can go. More importantly, transport activities can be quantified to examine whether MTS reagents are able to physically block the binding site or translocation pathway of the substrate. Using this technique,

several amino acids and transmembrane domains that are important in hASBT (human apical sodium-dependent bile acid transporter *SLC10A2*) mediated transport have been successfully mapped (Banerjee et al., 2005; Hussainzada et al., 2008; Khantwal and Swaan, 2008). However, one drawback of this technique is that sometimes, it is very difficult to obtain a cysteine free backbone which still retains the proper transport function.

(vii) Post-translational modification studies

In addition to interactions with key amino acids, transport function can also be affected by post-translational regulation, such as glycosylation and phosphorylation. As mentioned previously, there are three N-glycosylation sites in Oatp1a1, asparagines 124, 135, and 492, located at the extracellular loops 2 and 5. Single and multiple site-directed mutagenesis studies have shown that the extent of N-glycosylation controls mainly membrane targeting of Oatp1a1 and may also affect functional activity (Lee et al., 2003; Wang et al., 2008). The effect of phosphorylation of Oatp1a1 on transport function has also been studied. Glavy *et al.* (2000) have shown that phosphorylated Oatp1a1, as compared to unphosphorylated Oatp1a1, remains on the plasma membrane but has reduced transport activity (Glavy et al., 2000). Guo and Klaassen using the oocyte system, demonstrated that protein kinase C but not protein kinase A suppresses Oatp1a1 and Oatp1a4 mediated transport (Guo and Klaassen, 2001). A recent study has shown that protein kinase C mediated phosphorylation causes rapid internalization of OATP2B1 (Kock et al., 2010). These studies indicate that the phosphorylation

state is an important consideration when assessing the altered transport activities of OATP/Oatps in physiological and pathological conditions.

(2) Characteristics of substrates in OATP/Oatp-mediated transport.

Part (1) summarizes the structural requirements of transporter protein for the proper function of OATP/Oatp-mediated transport. As we know, OATP/Oatps are able to transport a wide spectrum of substrates. Therefore, studying the structural features of the substrates and how the substrates, inhibitors, and stimulators interact with the transporter is another effective approach to study the transport mechanism. In this following part, I will review the studies of the transport mechanism based on the transported substrates.

(i) Structural features of OATP/Oatp substrates

In general, most substrates of OATP/Oatps are amphipathic organic anions with a molecular weight of more than 450. OATP/Oatp substrates usually are bound to albumin under normal physiological conditions. These compounds are more likely to be secreted into bile as opposed to the smaller hydrophilic anionic compounds that are substrates of organic anion transporters (OATs) and are mostly secreted into urine (Hagenbuch and Meier, 2003). In recent years, researchers have been attempted to determine the common characteristics of Oatp/OATP substrates. One of the approaches many researchers have used is the substrate-based *in silico* modeling. Substrate-based modeling methods do not require prior knowledge of the transporter structure. They correlate the biological

activity of a series of substrates or inhibitors with their molecular or chemical properties. These models not only provide important information about the key structures of substrates, but also assist in the design or prediction of additional substrates. Two major methods used in this field are pharmacophore modeling and 3D-QSAR (quantitative structure-activity relationship) modeling. A pharmacophore is a certain spatial arrangement of structural features that are required for a particular biological function. Identification of a pharmacophore was originally designed to assist in the development of alternative or more active substrates or inhibitors for pharmaceutical purposes. This method has been widely used in the transporter field to study the binding of substrates. Pharmacophore models for Oatp1a1 and OATP1B1 have been studied and some molecular characteristics of OATP/Oatp substrates have been identified including hydrogen bond acceptors and hydrophobic features (Chang et al., 2005). Whereas a traditional pharmacophore model is able to distinguish an active structure versus an inactive structure, 3D-QSAR modeling method generates a mathematical equation quantitatively describing the bioactivity as a function of 3D spatial and chemical interactions to identify the important key structure features for substrate binding. Currently, the most widely used method of choice in 3D-QSAR studies is comparative molecular field analysis (CoMFA). It correlates both electrostatic and steric interactions with bioactivity (Chang and Swaan, 2006). A QSAR model for Oatp1a5 substrates has been generated and based on the derived model, new potential Oatp1a5 substrates have been identified and confirmed experimentally (Yarim et al., 2005). In addition, based on CoMFA analysis of nuclear receptor

ligands as competitive inhibitors of OATP1B1-mediated estradiol-17 β -glucuronide transport, the substrate binding site of OATP1B1 has been predicted to consist of a large hydrophobic middle section with basic residues at both ends (Gui et al., 2009).

(ii) Driving force of OATP-mediated transport

In addition to studying the substrate structures, the interactions between substrates and other molecules can also reveal information about the transport mechanism. However, the exact mechanism of the driving force of OATP/Oatp mediated transport is still unknown. OATP/Oatp-mediated transport has been shown to be independent of a sodium gradient (Kullak-Ublick et al., 1995; Noe et al., 1997; Walters et al., 2000). Moreover, there is evidence that OATP/Oatps may function as an exchanger. Intracellular GSH (Li et al., 1998; Li et al., 2000) and biocarbonate (Shi et al., 1995; Satlin et al., 1997) may play a role in Oatp1a1 and Oatp1a4-mediated transport. OATP2B1 has been shown to be more active and transport more substrates at low pH (Kobayashi et al., 2003). Later, Leuthold *et al.* (2009) have shown that most OATP/Oatps transport better at low pH due to a highly conserved histidine at the outside of TM 3 (Leuthold et al., 2009). For OATP1B1 and OATP1B3, it is generally accepted that they function as bidirectional carriers, but the role of GSH in transport mediated by members in OATP1B subfamily is controversial (Briz et al., 2006; Mahagita et al., 2007).

(iii) Modulators of OATP-mediated transport

In addition to the interactions of the transporter with both substrates and the “counter ions” that drive the exchange, many other modulators such as inhibitors and stimulators also affect OATP/Oatp mediated transport. In many occasions, OATP/Oatp substrates are also inhibitors of other OATP/Oatp substrates. Thus identifying the inhibitors of model substrates is often used as the first step of searching for new substrates. In addition, there are compounds, such as clotrimazole, that are able to stimulate transport of certain OATPs in a substrate dependent way (Gui et al., 2008). Therefore, both inhibitors and stimulators are important modulators for OATP/Oatp mediated transport. Moreover, because OATP/Oatps play very important roles in the disposition of xenobiotics, such as drugs and toxins, interactions of different molecules at the transporter level serve as one of the important underlying mechanisms of drug-drug or drug-food interactions that may cause the increase of drug toxicity or decrease of drug efficacy as described previously. Moreover, modulators could also be used as a way to increase transporter mediated drug uptake into target sites, such as cancer cells.

In summary, using *in silico* modeling methods combined with wet lab approaches, several basic features of OATP/Oatp substrates have been described. In general, many OATP/Oatp substrates are relatively large organic compounds that have a hydrophobic center with at least one hydrophilic group. Both hydrogen bonds and ionic bonds seem to play important roles in substrate binding and/or

translocations. However, the detailed mechanisms of OATP/Oatp-mediated transport still remain vague, and the general driving force of OATP/Oatps is still unknown. Compared to OATP/Oatps, the organic anion transporter (OAT) family has very similar structural and functional features, except the average molecular weight of substrates for OATs are smaller than for OATP (Hagenbuch, 2010). Similarly, numerous studies have investigated the structure-function relationships of OATs. Although the primary aim of this dissertation is to study the structure-function relationship of the OATP/Oatp family, it is always important to compare and contrast the subject of interest with the knowledge in the extended field. In the next section, I will briefly review some of the very basic features of the OAT family that serves as the background information of the primary research in chapter 5.

IV. BRIEF INTRODUCTION TO OAT

In the body, the liver and the kidney are the two major organs for the elimination of xenobiotics, including organic anions. Relatively small (molecular weight less than 400 to 500) and hydrophilic organic anions, also termed type-I organic anions, are primarily excreted via the kidney, whereas more hydrophobic, type-II organic anions, such as bile acids and glucuronide conjugates, are preferentially excreted by the liver (Sekine et al., 2006). It is not surprising that both liver and kidney have very complicated transporter systems facilitating the active transport of these compounds. Type-I organic anions are preferred substrates of the OAT family, whereas type-II organic anions are preferred

substrates for the OATP/Oatps family. The OAT family members are structurally similar to organic cation transporters (OCTs), and both OCT and OAT families belong to the *SLC22A* gene family. Similar to OATP/Oatps, both OATs and OCTs have very broad substrate spectra and members of both families are predicted to have 12-transmembrane domains. Members in the OAT family have multiple glycosylation sites localized in the first extracellular loop between transmembrane domains 1 and 2, and multiple phosphorylation sites in the intracellular loop between transmembrane domains 6 and 7 as well as in the carboxyl terminus (You, 2004). To date, the following members of the OAT family have been identified and functionally characterized: hOAT1/rOat1 (*SLC22A6*), hOAT2/rOat2 (*SLC22A7*), hOAT3/rOat3 (*SLC22A8*), hOAT4 (*SLC22A11*), rOat5 (*SLC22A9*), hURAT1 (*SLC22A12*) hOAT6/rOat6 (*SCL22A20*) and hOAT7 (*SLC22A9*).

Human OAT1 is the best studied member of the OAT family. It functions as an organic anion/dicarboxylate exchanger and is expressed in multiple tissues, primarily on the basolateral side of kidney proximal tubule cells (Sekine et al., 1997; Hosoyamada et al., 1999; Kojima et al., 2002). It can transport more than 100 different compounds, and mediates the active secretion of both endogenous chemicals and xenobiotics in kidneys (Sekine et al., 2006). The coding region of OAT1 has been shown to have low genetic diversity, and may not contribute substantially to inter-individual differences in renal elimination of xenobiotics

(Fujita et al., 2005). Similar to OATP/Oatps, glycosylation plays an important role in the targeting of OAT1 to the plasma membrane (Tanaka et al., 2004). Furthermore, a theoretical three-dimensional model was generated for human OAT1 (hOAT1) based on fold recognition with the crystal structure of the glycerol 3-phosphate transporter (GlpT) from *E. coli*. In combination with site-directed mutagenesis, two aromatic amino acids, Try230 in TM5 and Phe438 in TM10, have been identified to play important roles in substrate/transporter interactions (Perry et al., 2006). In addition, the chloride dependency of hOAT1 can be blunted by mutation of the conserved positively charged amino acid Arg466 in TM11 (Rizwan et al., 2007).

Among the OAT members in humans, OAT1, OAT3 and OAT4 are predominantly expressed in kidney, whereas OAT2 is abundantly expressed in liver (Hosoyamada et al., 1999; Cha et al., 2000; Sun et al., 2001). OAT2 has also been detected at the basolateral membrane of kidney proximal tubules (Enomoto et al., 2002b) whereas in rat, Oat2 is localized in the brush-border membrane (Kojima et al., 2002; Ljubojevic et al., 2007). Therefore, OAT2/Oat2 expression differs among species. Using the oocyte system, hOAT2 has been shown to be a sodium-independent multi-specific organic anion/dimethyldicarboxylate exchanger (Kobayashi et al., 2005). However, the detailed transport mechanism of OAT2/Oat2 is still unclear.

OAT3 and OAT1 have many similarities. First, OAT3 is primarily located on the basolateral membrane of proximal tubules (Race et al., 1999; Motohashi et al., 2002). Second, it is also a dicarboxylate/organic anion exchanger which moves organic anionic substrates into the cell (Race et al., 1999). In addition, knockout mice models of both Oat1 (Eraly et al., 2006) and Oat3 (Sweet et al., 2002) have been reported. Although both knockout mice showed no morphological abnormalities, the loss of renal organic anion transport was observed in both models. Several important amino acids have been identified using site-directed mutagenesis, including Arg454 and Lys370, that are essential for the anion specificity of rat Oat3 (Feng et al., 2001) and an aromatic pocket composed mainly of residues in TM7 that are in the translocation pathway of rat Oat3. The aromatic residues in this pocket may interact directly with substrates of rat Oat3 via hydrogen bonds and π - π interactions (Feng et al., 2002).

OAT4 is a human specific transporter localized primarily on the apical side of the proximal tubules (Ekaratanawong et al., 2004). It is also found in placenta and adrenocortical cells (Cha et al., 2000; Asif et al., 2005). Similar to OAT1 and OAT3, OAT4 is an organic anion/dicarboxylate exchanger (Ekaratanawong et al., 2004). Several histidines and glycines as well as N-glycosylation sites have been identified to be important for surface targeting of hOAT4 (Zhou et al., 2004a; Zhou et al., 2004b; Zhou et al., 2005). Very similar to Oatp1a1, it has been shown that surface expression and function of OAT4 is modulated through PDZ interaction with the network of PDZK1 and NHERF1 (Miyazaki et al., 2005).

OAT4 and URAT1 are the only two OAT members that contain PDZ domains. Interestingly, both transporters together with Oatp1a1 are all located on the brush border membrane of proximal tubule cells, suggesting that PDZ scaffold may be a mechanism of regulating the handling of organic anions on the apical side of proximal tubule cells.

Oat5 has been identified as a rodent specific transporter from mice and rat kidneys (Youngblood and Sweet, 2004; Anzai et al., 2005; Kwak et al., 2005). It is expressed in both males and female rodents and female mice has higher expression of Oat5 than male mice (Cheng and Klaassen, 2009). It has been shown that Oat5 can mediate sodium independent transport of estrone-3-sulfate and a number of drugs but the detailed transport mechanism is still unclear (Zhou and You, 2007).

URAT1/Urat1, another OAT family member, is a urate/organic anion exchanger that is expressed in the apical membrane of proximal tubule cells (Enomoto et al., 2002a). It is thought to play an important role in urate transport from the tubule lumen back into the proximal tubule cells for further reabsorption. The rat Urat1 has not been functionally characterized.

Oat6 is expressed predominantly in mouse olfactory mucosa (Monte et al., 2004; Kaler et al., 2006; Schnabolk et al., 2006). It is one of the few members of OATs/Oats which has no expression in kidney. Oat6 also acts as an organic

anion/dicarboxylate exchanger when expressed in *Xenopus* oocyte and Chinese hamster ovary (CHO) cells (Schnabolk et al., 2006). Human OAT6 has not been functionally characterized.

The liver specific OAT7 is expressed at the basolateral membrane of hepatocytes and was recently characterized as a multispecific organic anion transporter (Shin et al., 2007). In *Xenopus* oocytes, OAT7 transported estron-3-sulfate, dehydroepiandrosterone sulfate and the short chain fatty acid butyrate but none of the typical substrates of the organic anion or cation transporters. Furthermore, its transport was not affected by probenecid, the prototypical OAT inhibitor (Shin et al., 2007).

Among all members in the OAT family, the transport mechanisms of OAT1/Oat1 and OAT3/Oat3 have been mostly extensively studied. In general, they share a great amount of similarities with OATP/Oatp mediated transport. As mentioned previously, compared to OATP/Oatps, OATs/Oats preferentially transport smaller and more hydrophilic organic anions. However, sometimes, the substrate spectra for these two transporter families overlap. For example, perfluorooctanoate (C8), a compound widely used in industry, has been shown to be a substrate for hOAT1/rOat1 (Nakagawa et al., 2008), hOAT3/rOat3 (Nakagawa et al., 2008), hOAT4 (Nakagawa et al., 2009) as well as rOatp1a1 (Katakura et al., 2007; Yang et al., 2009). Kinetic analyses have shown that the K_m of rOat1 and rOat3 (51.0 μ M for Oat1 and 80.2 μ M for Oat3) (Nakagawa et

al., 2008) are much lower than the K_m of rat Oatp1a1 (162.2 μ M) (Yang et al., 2009) mediated C8 transport. Although both rat Oat and Oatp transporters are able to transport C8, Oat1 and Oat3 have higher affinity for C8 than Oatp1a1. We have known that the general substrates for OATP/Oatps have larger molecular weights than OAT/Oat substrates. If C8 fits better into the “binding pocket” of Oats, are Oatps better at transporting larger molecules that have similar structures? In addition to weight, what is the size preference of OATP/Oatp substrate? In the next section, I will introduce a series of compounds that have almost identical structures to C8, but with different chain length. These compounds will be used in the primary research in this dissertation described in chapter 5 to probe the spatial size of the “substrate binding pocket” of OATs/Oats and OATP/Oatps.

V. BRIEF INTRODUCTION TO PERFLUORINATED CARBOXYLATES

Perfluorinated carboxylates (PFCAs) are the fully fluorinated analogues of fatty acids. They share the same carbon backbones with regular fatty acids. However, instead of carbon-hydrogen bonds (C-H), PFCAs only have carbon-fluorine bonds (C-F). In general, the carbon chain lengths of the most commonly used PFCAs are from two carbons to eighteen carbons. Because of the electronegativity difference between carbon and fluorine (2.5 vs 4.0), C-F bonds are highly polar, and thus very strong. The strength of the C-F bonds makes PFCAs ideal chemicals to be used in industrial processes, especially in high temperature and corrosive environments.

Many studies have investigated the toxicological effects and environmental dispositions of PFCAs, especially C8 which is among the most widely used perfluorinated compound since the 1960s. Due to its extraordinary stability, C8 is persistent and bioaccumulates in the environment. Tests in rodents have demonstrated that C8 can activate peroxisome proliferator activated receptor α (PPAR α) and cause several negative effects, including increased β -oxidation of fatty acids, increased cytochrome P-450 (CYP450)-mediated reactions, and inhibition of the secretion of very low-density lipoproteins and cholesterol from liver (Kennedy et al., 2004). However, human exposure to perfluorinated compounds has not shown to produce significant clinical effects. Significant association of increased total cholesterol and uric acid serum levels with C8 serum levels has been reported recently (Costa et al., 2009). However, because most perfluorinated compounds used in industry, including C8, are highly resistant to degradation, it is important to understand their absorption, distribution, metabolism, and excretion, as well as potential health and target organ effects of these compounds.

Interestingly, all PFCAs have highly similar structural characteristics with many of the OATP/Oatp substrates, such as a hydrophobic center and negative potential at the hydrophilic head. The only difference between different PFCAs is the carbon chain length, and as a result, the size of the molecule. As a consequence, the hydrophobicity and lipophobicity will change with increased chain length. Table 1-3 summarizes the molecular weight and calculated chain

length of each PFCA. Based on the molecular weight, PFCAs with 9 carbons and above are more likely to be substrates for OATPs, whereas PFCAs with 8 carbons or less should be preferred substrates for OATs. Moreover, as the molecules get larger, the distance between the hydrophobic center and the hydrophilic head increases. If PFCAs with different chain lengths are indeed substrates for OATs, the predicted chain lengths may provide information with regard to the size of the “binding pocket” in OATs/Oats and OATP/Oatps. However, except for C8, there is no experimental evidence that other PFCAs are substrates of any OATs/Oats or OATP/Oatps before the studies presented in chapter 5 were reported.

Table 1-3: Summary of PFCAs with different chain lengths.

Carbon #	Name	Acronym	Molecular Formula	M.W	Calculated Length (Å)
C2	Trifluoroacetic acid		CF ₃ COOH	114.0	1.9
C3	Pentafluoropropionic acid		CF ₃ CF ₂ COOH	164.0	2.9
C4	Perfluorobutyric acid	PFBA	CF ₃ CF ₂ CF ₂ COOH	214.0	4.3
C5	Perfluoropentanoic acid	PFPA	CF ₃ (CF ₂) ₃ COOH	264.1	5.4
C6	Perfluorohexanoic acid	PFHxA	CF ₃ (CF ₂) ₄ COOH	314.1	6.8
C7	Perfluoroheptanoic acid	PFHpA	CF ₃ (CF ₂) ₅ CO ₂ H	364.1	7.3
C8	Perfluorooctanoic acid	PFOA	CF ₃ (CF ₂) ₆ COOH	414.1	8.8
C9	Perfluorononanoic acid	PFNA	CF ₃ (CF ₂) ₇ COOH	464.1	9.6
C10	Perfluorodecanoic acid	PFDA	CF ₃ (CF ₂) ₈ COOH	514.1	11.1
C11	Perfluoroundecanoic acid	PFUDA	CF ₃ (CF ₂) ₉ COOH	564.1	11.9
C12	Perfluorododecanoic acid	PFDDA	CF ₃ (CF ₂) ₁₀ COOH	614.1	13.4
C13	Perfluorotridecanoic acid	PFTTrDA	CF ₃ (CF ₂) ₁₁ COOH	664.1	14.3
C14	Perfluorotetradecanoic acid	PFTtDA	CF ₃ (CF ₂) ₁₂ COOH	714.1	15.8
C15	Perfluoropetadecanoic acid	PFPPDA	CF ₃ (CF ₂) ₁₃ COOH	764.1	16.8
C16	Perfluorohexadecanoic acid	PFHxDA	CF ₃ (CF ₂) ₁₄ COOH	814.1	18.2
C17	Perfluoroheptadecanoic acid	PFHpDA	CF ₃ (CF ₂) ₁₅ COOH	864.1	18.8
C18	Perfluorooctadecanoic acid	PFODA	CF ₃ (CF ₂) ₁₆ COOH	914.1	20.3

VI. CONCLUDING REMARKS FOR THE INTRODUCTION

In the past 15 years, since the discovery of the first Oatp, the field of OATP/Oatp-mediated transport has been developing rapidly. OATP/Oatps have the ability of transporting many structurally unrelated xenobiotics, including numerous drugs and toxins, and thus great attention has been drawn to the drug-drug or drug-food interactions that occur at the transporter level, as well as to the impact of OATPs on inter-individual variability of drug disposition. However, the studies of the more fundamental aspects, such as transport mechanism of OATP/Oatps, are still few in number and many principle questions remain: **1) How do OATP/Oatps transport substrates across the membrane; 2) Why can one single OATP/Oatp transport different substrates with unrelated structures; 3) What is the driving force of the transport; 4) What is the 3-dimensional structure of OATP/Oatps; and 5) Do OATP/Oatps function as multimers.** Although limited knowledge is available to answer these questions, the emerging research of many laboratories has begun to reveal some answers to these questions.

VII. SPECIFIC AIMS OF THIS DISSERTATION

To uncover the mechanisms of OATP/Oatp-mediated transport requires answers to very basic questions on how OATP/Oatps work at the molecular level, and what sub-domain(s) in the OATP/Oatp protein play key roles in initiating and completing the transport process, as well as what are the structural requirements of the active domain(s). Of course, much time and effort from many researchers

will be necessary to answer all of these questions. Thus, the overall goal of my research has been built upon previous findings, and has focused largely on identifying the key amino acids that play an important role in OATP/Oatp-mediated transport, and investigating the spatial size of the substrate binding pocket. In presenting this dissertation, I will defend three specific aims.

Specific aim 1 evaluates the hypothesis that **conserved positively charged amino acids play important roles in OATP1B1 transport function**. Based on a 3-dimensional structural model, several conserved positively charged amino acids have been suggested to be important for the function of OATP1B family members. To address this aim, site-directed mutagenesis was performed on selected amino acids and the resulting constructs were transiently expressed in HEK293 cells for functional analysis. Surface biotinylation and western blot were used to quantify total protein and surface expression of wild-type and mutated transporters. The purpose of this study was to determine whether these conserved positively charged amino acids are involved in substrate binding and /or substrate translocation.

Specific aim 2 evaluates the hypothesis that **quantifying transport activities of different substrates of chimeras between rat Oatp1a1 and Oatp1a4 in combination with site-directed mutagenesis should allow us to identify regions and/or individual amino acids that are important for Oatp1a4-mediated substrate recognition and/or transport**. To address this aim, chimeric proteins were made between Oatp1a1 and Oatp1a4 and transport of

digoxin, which is an Oatp1a4 but not an Oatp1a1 substrate, was used as a tool to investigate and identify the amino acids that are important in Oatp1a4 mediated transport function. In addition, the effects of mutations on transport of other common Oatp substrates such as taurocholate, DPDPE and estradiol-17 β -glucuronide were evaluated as well. The purpose of this study was to determine the critical region and/or residues that are important in Oatp1a4-mediated transport, and to further characterize the roles of these amino acids in the transport processes.

Specific aim 3 evaluates the hypothesis that **different rat renal organic anion transporters of the Oat and Oatp families selectively transport PFCAs, depending on chain lengths.** To address this aim, cDNA constructs of Oats or Oatp1a1 were transiently or stably transfected into HEK293 and CHO cells and the inhibitory effects of PFCAs with carbon chain lengths, from C2-C18, on transport of model substrates were evaluated. In addition, direct transport of the strongest inhibitors was quantified. The purpose of this study was to determine the size selectivity of Oats and Oatp1a1 in PFCA transport. Instead of altering the transporter structure, this is an approach using substrates of different sizes as rulers to probe the “substrate binding pocket” of intact transporters. Furthermore, these findings are also important in explaining the *in vivo* disposition patterns of PFCAs observed in rats.

CHAPTER 2

EXPERIMENTAL MATERIALS AND METHODS

I. CELL CULTURE

Human embryonic kidney (HEK293) cells were grown at 37°C in a humidified 5% CO₂ atmosphere in Dulbecco's Modified Eagle Medium High Glucose (Invitrogen, Carlsbad, CA), supplemented with 10% FBS (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA).

Chinese Hamster Ovary (CHO) cells (CHO-wild-type and CHO-Oatp1a1) were grown at 37 °C in a humidified 5% CO₂ atmosphere in Dulbecco's Modified Eagle Medium, containing 1 g/L D-glucose, 2 mM L-glutamine, 25 mM Hepes buffer, and 110 mg/l sodium pyruvate, supplemented with 10% FBS, 50 µg/mL L-proline, 100 U/mL penicillin, and 100 µg/mL streptomycin.

FlpIn™-HEK293 wild-type cells (Invitrogen, Carlsbad, CA) were maintained at 37 °C in a humidified 5% CO₂ atmosphere in Ham's F12/DMEM (1/1) medium supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL zeocin.

FlpIn™-HEK293 cells stably expressing rat Oat2 or stably transfected with pcDNA5/FRT empty vector were maintained at 37 °C in a humidified 5% CO₂

atmosphere in Ham's F12/DMEM (1/1) medium supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 400 µg/mL hygromycin B.

II. CONSTRUCTION OF AN OAT2 STABLE CELL LINE

Oat2 was cloned from kidney RNA of Sprague–Dawley rats. Briefly, total RNA was isolated from the frozen rat kidney using Trizol, following the manufacturer's instructions (Invitrogen Inc., Carlsbad, CA). Subsequently, RNA was reverse transcribed into single strand cDNA using Reverse Transcription System from Promega (Promega, Madison, WI). The open reading frame of rat Oat2 was amplified by PCR, using gene specific primers (Table 2-1) and a Phusion High-Fidelity PCR kit (Finnzyme, Espoo, Finland). The amplicon was then gel purified and subcloned into the pcDNA5/FRT vector for stable transfection into mammalian cells using *NheI* and *KpnI* restriction enzyme sites. The cDNA sequence was verified by sequencing both strands individually. FlpIn™-HEK293 cells were plated in 6-well plates without antibiotics, and then transfected as described in the manufacturer's instructions (Invitrogen, Carlsbad, CA). Twenty-four hrs after transfection, cells were re-plated onto 10 cm plates in medium supplemented with 400 µg/mL hygromycin B, for selection for two weeks with one subsequent 1:3 passage after one week. The pooled cells were tested for uptake of the model substrate [³H]-*p*-aminohippurate and used in PFCA transport studies. FlpIn™-HEK293 cells stably transfected with pcDNA5/FRT empty vector were used as a negative control.

Table 2-1: Primers used for cloning Oat2 from rat kidney

	Primer	Restriction site	Features
FWD 5'	ACCGGCTAGCGCCACCATGGGCTTCGAAGA CCTGCT	<i>NheI</i>	Kozak seq
RVS 3'	AGAGGGTACCCTAATGGTGATGGTGATGAT GCACATCTTCCTCCTGGGTA	<i>KpnI</i>	His tag

III. SITE-DIRECTED MUTAGENESIS OF OATP1B1

Human OATP1B1*1b was subcloned into the pcDNA5/FRT vector (Invitrogen, Carlsbad, CA) and was considered wild-type compared to the introduced mutations. Single amino acid mutations were introduced by site-directed mutagenesis using the QuickChange® system (Stratagene, La Jolla, CA), following the manufacturer's instructions. Briefly, a high fidelity DNA polymerase was used to replicate both strands of DNA plasmid using a set of primers with point mutations. Primers used in the mutagenesis reactions are listed in Table 2-2. After PCR cycles, the parental template DNA plasmids were digested by *DpnI*. The product of PCR was transformed into XL-1 Blue supercompetent cells, and ampicillin resistant colonies were subjected to sequencing to select for mutations. Plasmid DNA was prepared using HiSpeed Plasmid Midi or Maxi Kits (Qiagen, Germantown, MD), and both strands of all constructs were sequenced to confirm the presence of the designed mutations and the absence of additional spontaneous mutations.

IV. CONSTRUCTION OF CHIMERAS AND MUTANTS OF Oatp1a4

In order to avoid problems with different RNA stabilities due to different 3' untranslated regions, all constructs were cloned into the Oatp1a1 backbone. This Oatp1a1 backbone was constructed as follows: The Oatp1a1 cDNA (GenBank accession number NM_017111) in the pSPORT1 vector was cut with restriction enzymes *NcoI* and *BsmI*. The resulting overhangs were filled

Table 2-2: Primers used for site-directed mutagenesis of OATP1B1

Mutation		Primers (5'-3')
R181A	<i>forward:</i>	GTGTTTCATGGGTAATATGCTT GCT GGAATAGGGGAGACTCCC
	<i>reverse:</i>	GGGAGTCTCCCCTATTCC AGCA AGCATATTACCCATGAACAC
R181K	<i>forward:</i>	GTGTTTCATGGGTAATATGCTT AAAG GGAATAGGGGAGACTCCCATAG
	<i>reverse:</i>	CTATGGGAGTCTCCCCTATTCC TTT AAGCATATTACCCATGAACAC
R181H	<i>forward:</i>	CATGGGTAATATGCTT CAT GGAATAGGGGAGACTCCC
	<i>reverse:</i>	GGGAGTCTCCCCTATTCC ATGA AGCATATTACCCATG
R580A	<i>forward:</i>	CCACTCAATGGTTATAG GCA GCACTAGGAGGAATTCTAGC
	<i>reverse:</i>	GCTAGAATTCCTCCTAGTGCT TGCT ATAACCATTGAGTGG
R580K	<i>forward:</i>	CCACTCAATGGTTATAG AAAG CACTAGGAGGAATTCTAGCTCC
	<i>reverse:</i>	GGAGCTAGAATTCCTCCTAGTGCT TTT TATAACCATTGAGTGG
R580H	<i>forward:</i>	CCACTCAATGGTTATAG CAT GCACTAGGAGGAATTCTAGCTCC
	<i>reverse:</i>	GGAGCTAGAATTCCTCCTAGTGCT ATGT TATAACCATTGAGTGG
R57A	<i>forward:</i>	GTTCCATCATTCATATAGAA GCG GAGATTTGAGATATCCTCTTCTTG
	<i>reverse:</i>	CAAGAGAAGAGGATATCTCAAATCT CGCT TCTATATGAATGATGGAAC
R57K	<i>forward:</i>	GAA AAAG AGATTTGAGATATCCTCTTCTC
	<i>reverse:</i>	AAATCT CTTT TCTATATGAATGATGGAAC
K361A	<i>forward:</i>	GGTGCTTTTACTTATGTCTTC GCA TACGTAGAGCAACAGTATGG
	<i>reverse:</i>	CCATACTGTTGCTCTACGTAT TGCG AAGACATAAGTAAAAGCACC
K361R	<i>forward:</i>	TTC AGAT TACGTAGAGCAACAGTATGG
	<i>reverse:</i>	GTAT TCT GGAAGACATAAGTAAAAGCACC
K90A	<i>forward:</i>	GTGAGTTACTTTGGATCC GCA CTACATAGACCAAAGTTAATTGG
	<i>reverse:</i>	CCAATTAAC TTTGGTCTATGTAG TGCG GATCCAAAGTAACTCAC
K90R	<i>forward:</i>	GTGAGTTACTTTGGATCC CGA CTACATAGACCAAAGTTAATTGG
	<i>reverse:</i>	CCAATTAAC TTTGGTCTATGTAG TGCG GATCCAAAGTAACTCAC
H92A	<i>forward:</i>	GTTACTTTGGATCCAAACTAG GCT AGACCAAAGTTAATTGGAATCGG
	<i>reverse:</i>	CCGATTCCAATTAAC TTTGGTCT AGCT AGTTTGGATCCAAAGTAACTC
H92K	<i>forward:</i>	GAGTTACTTTGGATCCAAACTA AAAG AGACCAAAGTTAATTGGAATCGG
	<i>reverse:</i>	CCGATTCCAATTAAC TTTGGTCT CTT AGTTTGGATCCAAAGTAACTC
H92R	<i>forward:</i>	CTTTGGATCCAAACTA CGT AGACCAAAGTTAATTGGAATCGG
	<i>reverse:</i>	CCGATTCCAATTAAC TTTGGTCT ACGT AGTTTGGATCCAAAG
R93A	<i>forward:</i>	CTTTGGATCCAAACTACAT GCA CCAAAGTTAATTGGAATCGGTTG
	<i>reverse:</i>	CAACCGATTCCAATTAAC TTTGGT TGC ATGTAGTTTGGATCCAAAG
R93K	<i>forward:</i>	CTTTGGATCCAAACTACAT AAAC CAAAGTTAATTGGAATCGGTTG
	<i>reverse:</i>	CAACCGATTCCAATTAAC TTTGGT TTT ATGTAGTTTGGATCCAAAG

in with Klenow enzyme (Amersham Biosciences, Buckinghamshire HP7 9NA, England) and after dephosphorylation with calf intestinal alkaline phosphatase (Promega, Madison, WI, U.S.A.), the Oatp1a1 backbone was gel purified. This vector allowed cloning of blunt ended cDNAs between bases 89 and 2142 of the original Oatp1a1 construct resulting in identical 3' and 5' untranslated sequences.

To be able to detect all constructs with the same antibody, a 6-His tag was attached to the Oatp1a1 and Oatp1a4 open reading frame (ORF) by PCR amplification, with the primers Oatp1a1f90 and Oatp1a1rHis for Oatp1a1 and Oatp1a4f122 and Oatp1a4rHis for Oatp1a4 (Table 2-3). To reduce PCR related mutations, all amplifications were performed with the proofreading enzyme Pfu Turbo DNA Polymerase (Stratagene, La Jolla, CA). After amplification of the His-tagged Oatp1a1 and Oatp1a4 ORFs, they were cloned into the Oatp1a1 backbone. The correct orientation and sequence were confirmed by sequencing both strands on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

For the construction of the chimeras, an overlap PCR approach was used. In the first round of PCR, two overlapping fragments (chimf and chimr primers are at the same position on the two cDNA strands and thus overlap) were produced with templates and primers according to Tables 2-3 and 2-4. After gel-purification of the amplification products, they were used as templates for a second round of PCR, using the flanking primers (reverse and universal, Tables 2-3 and 2-4).

These final amplicons contained the multiple cloning site of the original pSPORT1 vector, and therefore were cut with the restriction enzyme *Sall* and *NotI*. After gel-purification of the digested fragments, they were directionally cloned into a *Sall-NotI* cut pSPORT1 vector and their correct sequence was verified by sequencing both strands.

To introduce single amino acid mutations, a similar overlap PCR approach was used. The middle primers (Table 2-3) contained the desired mutation and together with the flanking “reverse” and “universal” primers, the mutation was introduced after two rounds of PCR. All constructs were verified by sequencing both strands.

For transient transfection in HEK293 cells, all cDNA constructs containing His tags, including Oatp1a1, Oatp1a4, chimeric proteins and single point mutants of Oatp1a4 in the pSPORT1 vectors, were cut with the restriction enzymes *Sall*. The resulting overhangs were filled in with Klenow enzyme (New England Biolabs, Ipswich, MA). The cDNA inserts of Oatp1a1, Oatp1a4 and the mutants were then cut again with *NotI* and after dephosphorylation with calf intestinal alkaline phosphatase (New England Biolabs, Ipswich, MA), the inserts were gel purified and subcloned into pExpress-1 vector cut with *NotI* and *SmaI*.

Table 2-3: Primers used to prepare the different chimeras and mutants

Primer	Sequence (5' to 3')	
<i>Primers to introduce the His tag</i>		
Oatp1a1f90	GAAGAAACAGAGAAAAAGATTGCA	
Oatp1a1rHis	TTAATGGTGATGGTGATGATGCAGCTTCGTTTTTCAGT	
Oatp1a4f122	GGAAAATCTGAGAAAAGGGTTGCA	
Oatp1a4rHis	TCAATGGTGATGGTGATGATGGTCCTCCGTCACCTTC	
<i>Primers for chimeras</i>		<i>Chimera</i>
Reverse	CAGGAAACAGCTATGAC	All
Universal	TGTAAAACGACGGCCAGT	All
Chim1f	ATTTACATGCTTTTCATCCTTA	1/2
Chim1r	TAAGGATGAAAAGCATGTAAAT	1/2
Chim1af	TTACTGTCAAGAAAGCTGCA	3
Chim1ar	TGCAGCTTTCTTGACAGTAA	3
<i>Primers for mutations</i>		<i>Mutation</i>
Mut1for	AGTGTCTCCAGGTGAATGCATTTATC	F328V
Mut1rev	GATAAATGCATTCACCTGGAGAACACT	F328V
Mut2for	CTCCAGTTCAATGGCTTTATCAATTCA	A330G
Mut2rev	TGAATTGATAAAGCCATTGAACTGGAG	A330G
Mut3for	GCATTTATCAATAAATTTACCTTCATG	S334K
Mut3rev	CATGAAGGTAAATTTATTGATAAATGC	S334K
Mut4for	TCCACTGCTGAGGCAGTCTTCCTTATG	V353A
Mut4rev	CATAAGGAAGACTGCCTCAGCAGTGGA	V353A
Mut5for	ACTGCTGAGGTAATATTCCTTATGGGT	V354I
Mut5rev	ACCCATAAGGAATATTACCTCAGCAGT	V354I
Mut6for	ATGGGTCTTTATAGCTTACCTCCAATA	M361S
Mut6rev	TATTGGAGGTAAGCTATAAAGACCCAT	M361S

Table 2-4: Templates and primers used to construct the different chimeras

Chimera	Template	Forward primer 1 st PCR	Reverse primer 1 st PCR	Template 2 nd PCR	Forward primer 2 nd PCR	Reverse primer 2 nd PCR
1	Oatp1a1 Oatp1a4	Reverse Chim1f	Chim1r Universal	Amplification product of 1 st PCR	Reverse	Universal
2	Oatp1a4 Oatp1a1	Reverse Chim1f	Chim1r Universal	Amplification product of 1 st PCR	Reverse	Universal
3	Oatp1a1 Oatp1a4	Reverse Chim1af	Chim1ar Universal	Amplification product of 1 st PCR	Reverse	Universal

V. TRANSIENT TRANSFECTION OF HEK293 CELLS

Twenty-four hrs before transfection, HEK293 cells were harvested by trypsinization and re-plated at 250,000 cells/well in 24-well plates (coated with 0.1 mg/ml poly-D-lysine). The transfection mixture consisted of 0.8 µg of plasmid DNA and 2µL LipofectamineTM 2000 (Invitrogen, Carlsbad, CA). The transfection was performed according to the manufacture's protocol and the transfected cells were incubated at 37°C for 48 hrs before use.

VI. PROTEIN EXPRESSION IN STABLE CELL LINES

For Oatp1a1 and Oat2 stable cell lines, cells were plated at 40,000 cells per well on 24-well plates and 48 h later medium was replaced with medium containing 5 mM sodium butyrate to induce nonspecific gene expression (Palermo et al., 1991). After another 24 h in culture, the cells were used for uptake experiments or surface biotinylation.

VII. PROTEIN EXPRESSION IN *X. LAEVIS* OOCYTES:

For in vitro synthesis of cRNA, the cDNA clones in pSPORT-1 vector were linearized with *NotI* and capped cRNA was synthesized using the mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX) according to the manufacture's protocol. *X. laevis* oocytes were prepared and handled as described previously (Hagenbuch et al., 1991; Hagenbuch et al., 1996). Briefly, frogs were anaesthetized by immersion for 15 min in a 0.17% solution of ethyl *m*-aminobenzoate (MS-222). Oocytes were

removed and incubated at room temperature for 3 hrs in Ca^{2+} -free OR-2 solution, supplemented with 2 mg/ml collagenase (Sigma type I). They were then washed in modified Barth's solution, consisting of 88mM NaCl, 1mM KCl, 2.4mM NaHCO_3 , 15mM HEPES-NaOH, pH 7.6, 0.3mM $\text{Ca}(\text{NO}_3)_2 \cdot (4\text{H}_2\text{O})$, 0.41mM $\text{CaCl}_2 \cdot (6\text{H}_2\text{O})$, 0.82mM $\text{MgSO}_4 \cdot (7\text{H}_2\text{O})$, 10 units/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin. Stage V and VI oocytes were selected for further experiments. After an overnight incubation at 18 °C, healthy oocytes were injected with 50 nl water or 5 ng of the respective cRNA.

VIII. TRANSPORT ASSAY (*X. LAEVIS* OOCYTES):

After 3 days in culture at 18 °C, transport of radiolabeled substrate [^3H]-digoxin (PerkinElmer Life Sciences, Boston, MA) was quantified at 25 °C in an oocyte uptake buffer containing 100 mM choline chloride, 2 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM Hepes/Tris, pH 7.5. Oocytes were washed once in oocyte uptake buffer. Between 5 and 10 oocytes were then incubated at 25 °C in uptake buffer containing radiolabeled substrates. After the indicated time interval, the uptake was terminated by the addition of 1 ml of ice-cold uptake buffer. Furthermore, oocytes were additionally washed 3 times in 10 ml of cold buffer. Single oocytes were then dissolved in 1 ml of 10% (w/w) sodium dodecyl sulfate. After addition of 5 ml of scintillation fluid (Opti-FluorTM Packard Instrument International, S.A., Zurich, Switzerland) the oocyte-associated radioactivity was quantified in a Packard Tri-CarbTM 2200 CA liquid scintillation analyzer.

Kinetic analysis of digoxin was determined at 15 min which was within the initial linear range. The obtained values, corrected for the values of the water injected oocytes, were fitted to the Michaelis-Menten equation using non-linear regression analysis.

IX. TRANSPORT ASSAY (MAMMALIAN CELLS)

After washing the cells three times with prewarmed (37°C) uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.5 mM CaCl_2 , 5 mM glucose, and 12.5 mM HEPES, pH 7.4), 200 μL uptake buffer containing radiolabeled substrates with sufficient unlabeled compound to achieve the indicated concentrations, were added to initiate transport. After incubating for the indicated time periods, transport was terminated by four washes with ice-cold uptake buffer. Cells were lysed with 300 μL 1% Triton X-100 at room temperature for 30 min. Two hundred microliters of cell lysate were transferred to 24-well scintillation plates (Perkin Elmer, Shelton, CT) and radioactivity was quantified after adding Optiphase Supermix scintillation cocktail (Perkin Elmer) in a MicroBeta liquid scintillation counter. For unlabeled PFCA substrates, final concentrations in cell lysates were analyzed using LC-MS/MS. The remaining 100 μL cell lysate were used to determine the protein concentration using the BCA^{TM} Protein Assay (Pierce). All transport activities were corrected for total protein concentration.

Kinetic analyses were all determined within the initial linear time range. Transport of radiolabeled estradiol-17 β -glucuronide was quantified from 1 μ M to 50 μ M; transport of radiolabeled estrone-3-sulfate was quantified from 0.05 μ M to 2 μ M; transport of radiolabeled sulfobromophthalein was quantified from 0.05 μ M to 3 μ M; transport of radiolabeled taurocholate was quantified from 5 μ M to 200 μ M; transport of radiolabeled digoxin was quantified from 0.1 μ M to 5 μ M and transport of PFCAs was quantified from 10 μ M to 300 μ M. Transporter-specific uptake was obtained by subtracting the uptake into empty vector-transfected cells or wild-type cells from the uptake into transporter expressing cells. Michaelis-Menten type nonlinear curve fitting was carried out to obtain estimates of the maximal uptake rate (V_{\max}) and the apparent affinity constant (K_m) (Graphpad Prism, GraphPad Software Inc., La Jolla, CA).

X. SURFACE BIOTINYLATION IN HEK293 CELLS:

Forty eight hrs after transfection, HEK293 cells were washed with ice-cold phosphate-buffered saline $\text{Ca}^{2+}/\text{Mg}^{2+}$ (PBS-CM; 138mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4 , 9.6mM Na_2HPO_4 , 1mM MgCl_2 , 0.1mM CaCl_2 , pH7.4) and then treated with 1mg/ml membrane-impermeable biotinylating agent sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin; Pierce, Rockford, IL) at 4°C for 1 hr with shaking. Subsequently, cells were washed three times with ice cold PBS-CM containing 100mM glycine to remove the remaining labeling reagent. The cells were then lysed in 300 μ L lysis buffer (150mM NaCl, 10mM Tris-HCl,

1mM EDTA, 1% Triton X-100 and 0.1% SDS, pH7.5) containing protease inhibitors (Complete, Roche Applied Science, Indianapolis, IN) at 4°C with constant agitation for 1 hr. Following centrifugation at 10,000 x g (4°C) for 2 min, 50µL of NeutrAvidin (Pierce) beads were added to 250µL of cell lysate supernatant and incubated at room temperature for 1 hr with constant agitation. Beads were then washed 4 times with lysis buffer, and the biotinylated proteins were recovered from the beads by incubating with 2xLaemmli buffer with 50mM DTT for 30 min at room temperature.

XI. SDS-PAGE AND WESTERN BLOT

Forty micro liters of surface proteins were separated on 8% SDS-PAGE minigels at 150V for 1 hr and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) for 1 hr at 100V. The membrane was blocked for 1 hr with 5% non-fat dry milk in PBS at room temperature, followed by overnight incubation of anti-OATP1B1 antibody (1:2500 dilution) at 4°C. After washing with 0.1% PBS/Tween-20 3 times for 10 min each, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) for 1 hr at room temperature in 2.5% milk in PBS (1:10,000 dilution). Following extensive washing with 0.1% PBS/Tween-20, the secondary antibody was detected using the ECL kit (AmershamTM, Buckinghamshire, UK). Protein loading was normalized using an antibody against the plasma membrane marker Na⁺/K⁺ ATPase α subunit (Abcam, Cambridge, MA) (1: 5000 dilution). Horseradish peroxidase-conjugated goat anti-

mouse IgG (Pierce) was used as secondary antibody (1:10,000 dilution). Immunoblots were developed using the ECL plus kit (AmershamTM). The intensity of protein bands was quantified using ImageJ (<http://rsbweb.nih.gov/ij/>). Band intensities at 5-6 different exposure times were quantified to ensure that the signal was within the linear range of the film.

XII. LC-MS/MS ANALYSIS FOR PFCAS

For single concentration uptake and time dependency studies, Waters ACQUITY ultra performance LC system with a Waters Quattro Premier XE triple quadrupole mass spectrometer with an ESI source (Waters, Milford, MA) were used. The entire LC-MS system was controlled by MassLynx 4.1 software. Chromatographic separations were performed with an ACQUITY UPLC C18 column (1.7 μ m, 50 mm \times 2.1 mm ID) (Waters, Milford, MA). The mobile phase consisted of 35% H₂O and 65% acetonitrile, at a total flow rate of 0.3 mL/min. MRM transitions 363 \rightarrow 319 amu, 413 \rightarrow 369 amu, 463 \rightarrow 419 amu, 513 \rightarrow 469 amu were chosen for C7, C8, C9 and C10 quantification. C7 was used as internal standard for C8, C9 and C10 quantification, whereas C8 was used as an internal standard for C7 quantification. Samples were prepared by adding 60 μ L cell lysate into 120 μ L acetonitrile containing internal standard. The tubes were then vortexed vigorously for 5 sec followed by centrifugation at 25,000 \times g for 20 min. One hundred and fifty microliters of supernatant were then transferred to a new clean polypropylene tube and stored at -20°C until analyzed.

For transporter kinetic studies, the instrument used was the Sciex API 5000 mass spectrometer (Applied Biosystems / MDS-Sciex, Foster City, CA). The Turbo Ion Spray (pneumatically assisted electrospray ionization) in negative ion mode was used. Separation of the compounds were completed on a Mac-Mod ACE ® C-18, 5 µm, 100 x 2.1 mm i.d. HPLC column with a gradient flow rate of 0.250 mL/min. Mobile phase was acetonitrile and 2 mM ammonium acetate solution in reagent grade water. The initial mobile phase was 25% acetonitrile and 75% ammonium acetate solution. The concentration was held for 1.0 min. Between 1.0 min and 4.0 min the composition was changed to 10% 2 mM ammonium acetate solution and 90% acetonitrile. The 90% acetonitrile composition was held until 7.00 min into the run. Between 7 min and 7.80 min the acetonitrile concentration was decreased to 25% and held until 10.5 min until re-equilibration of the HPLC columns was completed. Transition ions were monitored as follows: C7: 363 → 319 amu; C8: 413 → 369 amu; C9: 463 → 419 amu ; C10: 513 → 469 amu; Internal Standard: dual ¹³C-labeled C8: 415 → 370 amu. Samples were prepared by the addition of 25 µL of cell lysate to the polypropylene 1.8 mL conical tip tube containing the internal standard matrix matched blank buffer. Standards were introduced to similar tubes and matrix matched blank buffer (25µL) of either CHO lysate or HEK293 lysate were pipetted as appropriate. To the tube containing the 25 µL of cell lysate, 175 µL of a solution containing 5% of 2 mM ammonium acetate and 95% acetonitrile was added (total volume 200 µL). The tube was vortexed vigorously for 5 sec followed by centrifugation at 2500 x g. An 80 µL aliquot of supernatant was transferred to a new

clean 100 μ L polypropylene vial; the vial was capped with a polypropylene cap and placed in the auto sampler for injection into the LC-MS/MS system. A 4 μ L injection was used for the analysis.

XIII. STATISTICAL ANALYSIS

Statistical significance was analyzed using a two-tailed, unpaired Student's t test (GraphPad Prism, GraphPad Software Inc., La Jolla, CA). Data were considered statistically significant at $p < 0.05$.

CHAPTER 3

SEVERAL CONSERVED POSITIVELY CHARGED AMINO ACIDS ARE IMPORTANT FOR OATP1B1 FUNCTION

SPECIFIC AIM AND HYPOTHESIS

Chapter 3 will address specific aim 1 and will evaluate the hypothesis that **conserved positively charged amino acids play important roles in OATP1B1 transport function.** Based on a 3-dimensional structural model, several conserved positively charged amino acids have been suggested to be important for the function of OATP1B family members. The purpose of this study is to **determine whether these conserved positively charged amino acids are involved in substrate binding and /or substrate translocation using site-directed mutagenesis.**

I. INTRODUCTION

OATP1B1 is a transport protein expressed at the basolateral membrane of human hepatocytes (Abe et al., 1999; Hsiang et al., 1999; Konig et al., 2000). It belongs to the organic anion transporting polypeptide superfamily (OATP, gene symbol *SLCO*) (Hagenbuch and Meier, 2004), and mediates the sodium-independent transport of a wide range of structurally unrelated amphipathic organic compounds (Konig et al., 2006; Hagenbuch and Gui, 2008). Due to its broad substrate spectrum, OATP1B1 is important for hepatic clearance of many drugs and other xenobiotics.

Therefore, understanding the transport mechanisms of OATP1B1 is essential to explain and prevent potential adverse drug interactions occurring at the transporter level.

Naturally occurring polymorphisms have provided some insight into the structure-function relationship for OATP1B1. OATP1B1*1b (388A>G, N130D) and OATP1B1*5 (521T>C, V174A) are the two most frequently observed polymorphisms (Tirona et al., 2001; Nozawa et al., 2002; Konig et al., 2006). N130D does not affect transport of most substrates or surface expression of the protein (Tirona et al., 2001; Iwai et al., 2004; Kameyama et al., 2005), whereas V174A alone or together with N130D (OATP1B1*15) decreases clearance and increases plasma concentration of pravastatin, simvastatin, ezetimibe, SN-38 and bilirubin (Nishizato et al., 2003; Ieiri et al., 2004; Niemi et al., 2004; Nozawa et al., 2005; Zhang et al., 2007; Han et al., 2008; Neuvonen et al., 2008; Oswald et al., 2008; Pasanen et al., 2008; van der Deure et al., 2008). In vitro studies using transiently transfected cell lines suggest that the decreased transport activity of OATP1B1*15 may be due to decreased protein expression on the cell surface (Kameyama et al., 2005) or due to decreased turnover number of the mutated transporter (Iwai et al., 2004). Structurally, OATP1B1 shares 80% amino acid sequence identity with OATP1B3, another transporter in the same subfamily. Functionally, OATP1B1 and OATP1B3 exhibit overlapping substrate spectra in addition to their own specific substrates (Konig et al., 2006; Hagenbuch and Gui, 2008). Therefore, construction of chimeric proteins

between OATP1B1 and OATP1B3 is another strategy to study structure-function relationship for both transporters. Using this technique, it has been shown that three amino acid residues within transmembrane domain 10 in OATP1B3 are important for recognition and/or translocation of CCK-8, an OATP1B3 specific substrate (Gui and Hagenbuch, 2008). In addition, using the same strategy, the eighth, ninth (Miyagawa et al., 2009) and tenth (Gui and Hagenbuch, 2009) transmembrane domain of OATP1B1 have been shown to affect the transport of estrone-3-sulfate and/or estradiol-17 β -glucuronide.

Due to the lack of crystal structure information, comparative modeling has been used to predict the structure of OATPs. Because OATPs are predicted to have 12-transmembrane domains, which is common to members of the major facilitator superfamily (MFS), the known crystal structures of two membrane transporters in the MFS were used as templates to generate a 3D structural model for OATP1B3 (Meier-Abt et al., 2005). Based on this model, a putative binding pocket within an aqueous pore that contains several positively charged amino acid residues that are conserved throughout the OATP1 family was proposed (Meier-Abt et al., 2005). A recent study has confirmed in OATP1B3 that K41 and R580 in transmembrane domains are important for BSP transport (Glaeser et al., 2010). However, the roles of these positively charged amino acids in OATP1B1 mediated transport are still unknown.

Therefore in this study, we performed site-directed mutagenesis and characterized the mutant proteins with respect to surface expression and transport function to determine the significance of these conserved positively charged amino acids for OATP1B1.

II. RESULTS AND DISCUSSION

2.1 Expression and function of alanine mutants

To determine the functional effects of the individual conserved positively charged amino acids facing the putative binding pocket (Meier-Abt et al., 2005), we performed site-directed mutagenesis and changed amino acid residues at the positions indicated in Figure 3-1; R181 and R580 in predicted transmembrane domains 4 and 11, R57 and K361 at the predicted extracellular side, and K90, H92 and K93 at the predicted intracellular side of OATP1B1. Initially, we individually replaced all seven amino acids with alanines and transiently expressed these mutants in HEK293 cells. We purified membrane proteins using surface biotinylation and performed western blot analysis using an anti-OATP1B1 antibody. As demonstrated in Figure 3-2, all OATP1B1 constructs were detected at the cell surface at different expression levels. Na⁺/K⁺ ATPase, a membrane protein naturally expressed in all HEK293 cells, was used as loading control. Most alanine mutants were able to express normally on the cell surface, except for R580A which was only expressed at about 30% of the control. We observed this low expression level also in total cell lysates (data not shown), indicating that this mutation may alter protein synthesis, folding or stability.

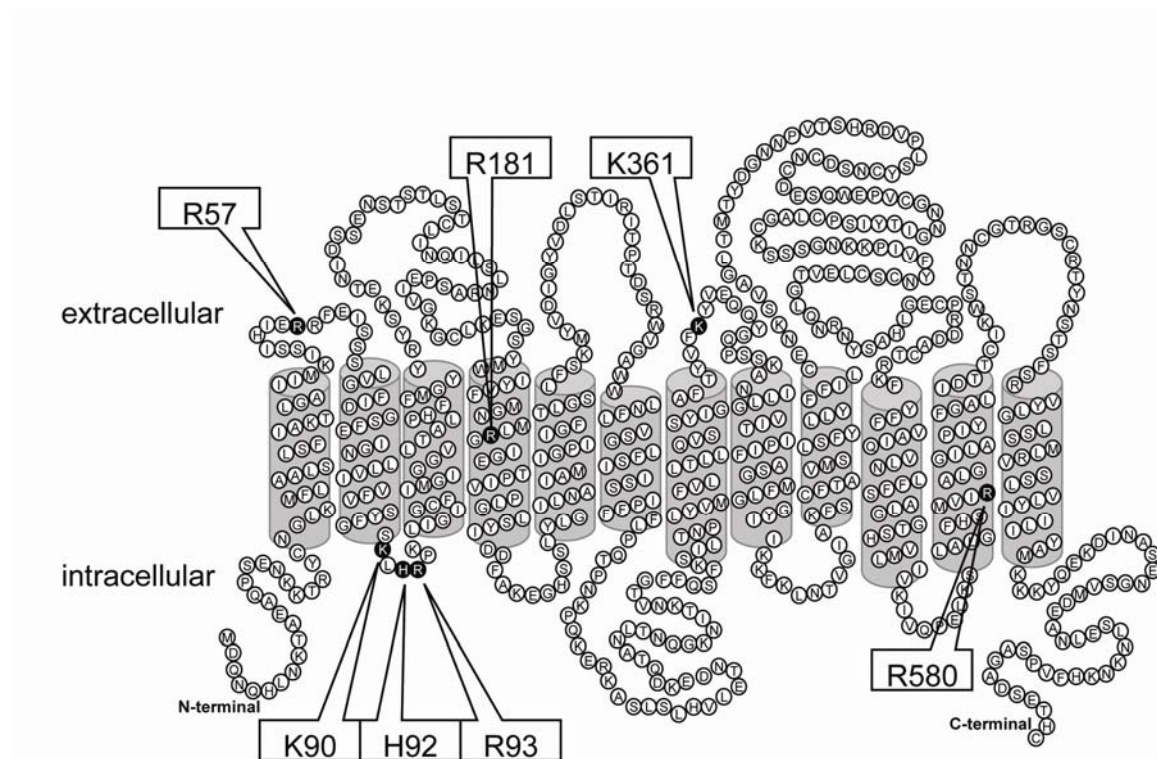


Figure 3-1 Predicted topological model of human OATP1B1 with mutation sites

Topological structure of OATP1B1 was predicted by TMPred

(http://www.ch.embnet.org/software/TMPRED_form.html). Amino acids mutated in this study are indicated in black and the positions are listed in the boxes pointing to these residues.

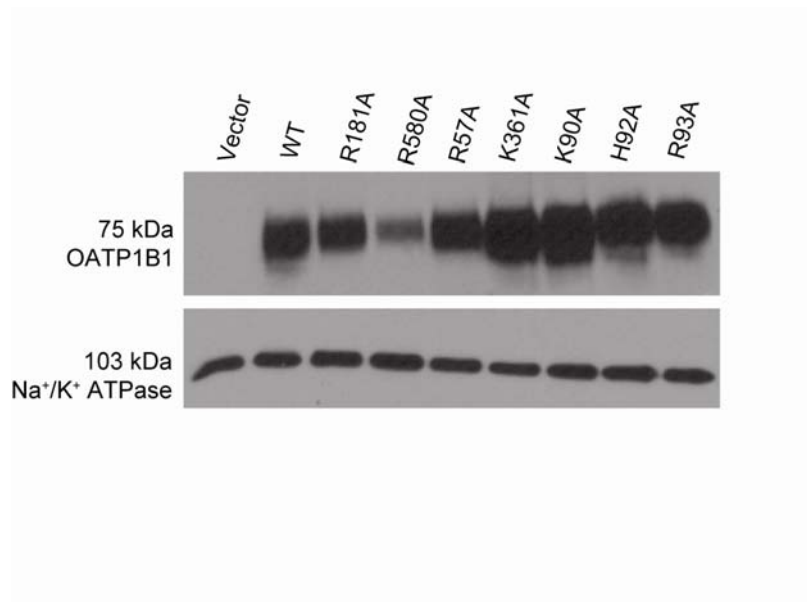


Figure 3-2 Surface expressions of alanine mutants in HEK293 cells

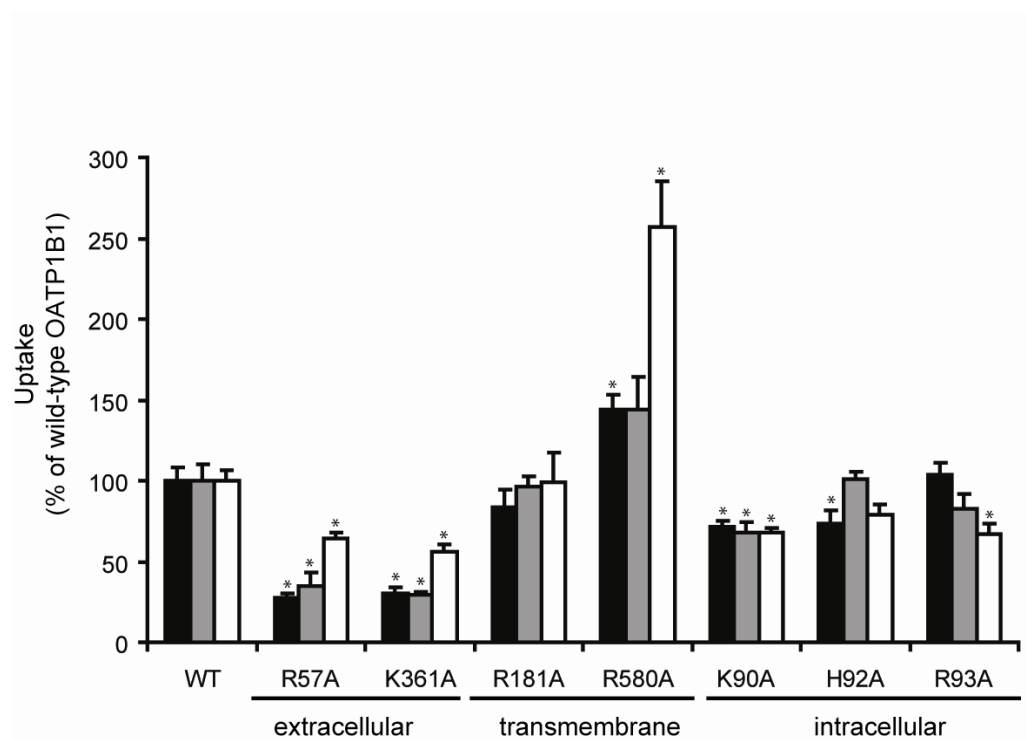
Representative western blots of surface-biotinylated proteins detected with a polyclonal anti-OATP1B1 antibody (75kDa). HEK293 cells transfected by empty vector were used as negative controls. The same blot was also probed with an antibody against the plasma membrane marker Na⁺/K⁺-ATPase α subunit (103 kDa) as a protein loading control.

Given that OATP1B1 is a multispecific transporter (Hagenbuch and Gui, 2008) and that for certain substrates multiple substrate binding sites have been suggested (Tamai et al., 2001; Noe et al., 2007; Hagenbuch and Gui, 2008), we determined the transport activity of each mutant by quantifying uptake of the model substrates [^3H]-estradiol-17 β -glucuronide, [^3H]-estrone-3-sulfate, and [^3H]-sulfobromophthalein (BSP). Quantified values were normalized to whole cell protein as well as to surface expressed OATP. First, we quantified uptake of 1 μM estradiol-17 β -glucuronide. HEK293 cells transfected with wild-type OATP1B1 transported 63 ± 8 pmol/mg total protein/min estradiol-17 β -glucuronide which was 70 fold higher than empty vector transfected HEK293 cells (0.9 ± 0.2 pmol/mg total protein/min). Net uptake was considered as wild-type OATP1B1-mediated transport and set to 100%. This high signal to background ratio allowed us to compare transport activities of mutants to wild-type OATP1B1. Figure 3-3 shows that transport of estradiol-17 β -glucuronide (black bars) by the two mutants at the extracellular side, R57A and K361A, was reduced to about 30% of wild-type OATP1B1. Transport by the transmembrane domain mutant R181A was not different from wild-type OATP1B1, whereas transport by R580A was slightly increased. Of the three intracellular mutants, K90A and H92A showed slightly decreased transport whereas transport by R93A was normal (Figure 3-3). For estrone-3-sulfate at a concentration of 0.1 μM , HEK293 cells transfected with wild-type OATP1B1 transported 8.3 ± 0.8 pmol/mg total protein/30s, whereas empty vector transfected HEK293 cells transported only 0.14 ± 0.02 pmol/mg total protein/30s. This resulted in a net uptake of 8.2 ± 0.9 pmol/mg total protein/30s

which after correction for surface expression was set to 100 %. Compared to estradiol-17 β -glucuronide, we observed similar results for the transport of estrone-3-sulfate by the alanine mutants (Figure 3-3, grey bars). The results with BSP, another high-affinity substrate for OATP1B1 (Cui et al. 2001, Kullak-Ublick et al. 2001), are shown in Figure 3-3 with white bars. Uptake of 0.1 μ M BSP by wild-type OATP1B1 amounted to 13.1 \pm 2.0 pmol/mg total protein/min, whereas empty vector transfected HEK293 cells transported 1.2 \pm 0.4 pmol/mg total protein/min. This resulted in a net uptake of 11.9 \pm 2.0 pmol/mg total protein/min, which was set to 100%. Compared to the previous two substrates, BSP transport was not as strongly affected by alanine mutations at the two extracellular sites R57 and K361. Uptake by the transmembrane R181A was comparable to estradiol-17 β -glucuronide and estrone-3-sulfate, whereas uptake by R580A was markedly increased. Mutations of the intracellular residues K90 and H92 to alanine resulted in BSP uptake similar as for estradiol-17 β -glucuronide and estrone-3-sulfate, whereas an alanine at position R93 affected BSP transport more than the other two substrates. These results were a first hint for substrate specific effects of the different amino acid replacements.

Figure 3-3 Uptake of estradiol-17 β -glucuronide estrone-3-sulfate and sulfobromophthalein mediated by OATP1B1 and alanine mutants after normalization for surface expression.

Uptake of 1 μ M [3 H]-estradiol-17 β -glucuronide (black bar) and 0.1 μ M [3 H]-sulfobromophthalein (BSP, white bar) was quantified at 37°C for 1min (initial linear rate condition); uptake of 0.1 μ M [3 H]-estrone-3-sulfate (gray bar) was quantified at 37°C for 30 sec (initial linear rate condition) with HEK293 cells expressing empty vector, OATP1B1, or alanine mutants. The net uptakes were obtained by subtracting the uptake of empty vector transfected cells from the uptake of OATP1B1 or mutant transfected cells. The results were normalized for surface protein expression and presented as percentage of wild-type OATP1B1 (WT). Each bar is the mean \pm standard error (SE) of five independent experiments. Asterisks (*) indicate significant difference from wild-type OATP1B1. *, p<0.05.



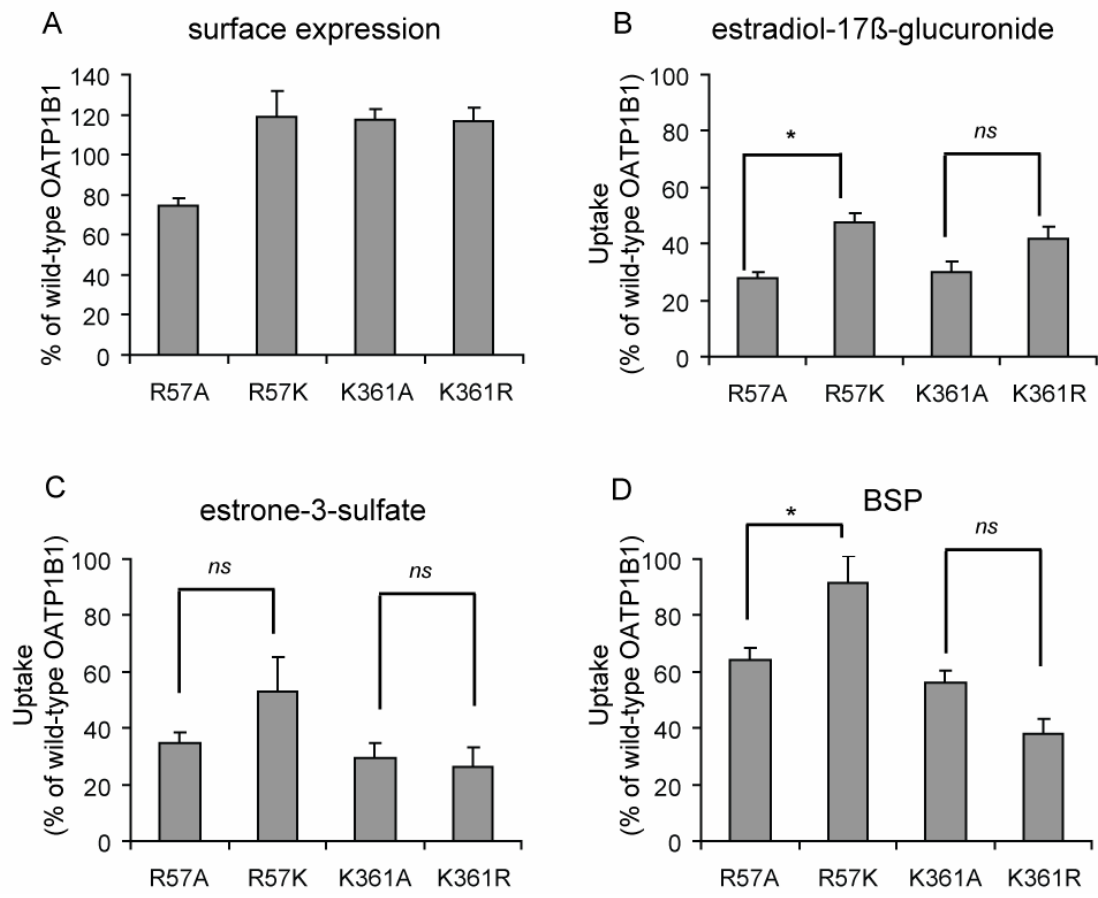
2.2 Expression and function of mutants on the extracellular side

In order to test whether re-introduction of a positively charged amino acid residue at positions 57 and 361 would rescue transport function, we constructed mutants that contained a lysine at 57 (R57K) or an arginine at 361 (K361R). Both mutants were expressed at the surface normally (Figure 3-4A). Re-introduction of the positively charged lysine at position 57 (R57K) increased transport of estradiol-17 β -glucuronide (Fig. 3-4B) and BSP (Fig. 3-4D) as compared to the alanine mutant. Estrone-3-sulfate transport was slightly increased, but the increase did not reach statistical significance (Fig. 3-4C). Compared to the alanine mutant, re-introduction of a positive arginine at position 361 (K361R) did not affect transport of any of the three tested substrates (Fig. 3-4B, C, D). These results suggest that at least for estradiol-17 β -glucuronide and BSP, a positively charged residue at position 57 is important, whereas at position 361, the lysine is required and cannot be replaced by the positively charged arginine.

It is important to emphasize that all these experiments were performed at a single time point and a single concentration within the initial linear range. Although such experiments can yield important information about changes in the transport activities of the involved proteins, kinetic analyses are needed to make conclusions about potential changes in the apparent affinities or V_{\max} values. Therefore, we determined the kinetic parameters for wild-type OATP1B1 and the extracellular mutants for estradiol-17 β -glucuronide, estrone-3-sulfate, and BSP. Although two

Figure 3-4 Surface expression and transport function of extracellular R57 and K361 mutants.

(A) Quantification of surface expression of R57 and K361 mutants normalized to loading control Na^+/K^+ -ATPase presented as a percentage of wild-type OATP1B1 (WT). Each bar is the mean \pm standard error (SE) of five independent experiments. Uptake of $1\mu\text{M}$ [^3H]-estradiol-17 β -glucuronide (B), $0.1\mu\text{M}$ [^3H]-estrone-3-sulfate (C) or $0.1\mu\text{M}$ [^3H]-sulfobromophthalein (BSP) (D) mediated by R57 and K361 mutants was quantified at 37°C under initial linear rate conditions and presented as % of wild-type after correction for surface expression. Each bar is the mean \pm standard error (SE) of five independent experiments. *, $p<0.05$; ns, no significant difference.



binding sites were identified for OATP1B1 mediated estrone-3-sulfate transport (Tamai et al., 2001; Noe et al., 2007), we only studied the high-affinity component because transport at low concentrations was affected by the mutations. The data in Table 3-1 summarizes the kinetic experiments. Apparent K_m values obtained for wild-type OATP1B1 were within the published values for estradiol-17 β -glucuronide (Konig et al., 2000; Cui et al., 2001; Tamai et al., 2001; Hirano et al., 2004; Gui et al., 2008), estrone-3-sulfate (high-affinity, low-capacity binding site) (Hirano et al., 2004; Noe et al., 2007) and BSP (Cui et al., 2001; Kullak-Ublick et al., 2001). With respect to estradiol-17 β -glucuronide, both alanine mutants, R57A and K361A, had lower affinities as compared to wild-type OATP1B1. The apparent K_m values increased almost 5 fold for R57A from 5.7 to 27.1 μ M and moderately for K361A to 14.5 μ M. Restoring the positive charges resulted in a reversal of the effects for position 57. The apparent K_m value for R57K (8.6 μ M) was close to the K_m value for wild-type OATP1B1 (5.7 μ M), whereas the apparent K_m value of K361R remained elevated like the K_m value obtained for K361A. These data suggest that a positively charged amino acid at position 57 is important for a normal estradiol-17 β -glucuronide transport, whereas at position 361 the lysine is required. The corresponding V_{max} values did not change as much as the apparent K_m values (Table 3-1), and thus the transport efficiencies (V_{max}/K_m) for all four mutants were mainly affected by the K_m values and clearly decreased to less than 50 % of wild-type OATP1B1 (Table 3-1). However, reintroducing a positively charged residue at position 57 increased transport efficiency from 27 to 50 % of wild-type, whereas both mutants at position

Table 3-1: Kinetic parameters of wild-type OATP1B1 and extracellular mutants

Substrate	Mutant	K _m (μ M)	V _{max} (pmol/mg protein per min)	V _{max} /K _m (μ l/mg protein per min)	% of WT
estradiol-17 β -glucuronide	WT	5.7 \pm 0.6	271.2 \pm 17.4	47.5 \pm 3.5	100 \pm 7
	R57A	27.1 \pm 1.8 *	347.7 \pm 20.4 *	12.8 \pm 0.7 *	27 \pm 1
	R57K	8.6 \pm 0.3 *	205.4 \pm 14.9	23.8 \pm 1.1 *	50 \pm 2
	K361A	14.5 \pm 2.0 *	213.7 \pm 10.0	14.8 \pm 1.3 *	31 \pm 3
	K361R	11.9 \pm 2.6 *	224.1 \pm 24.2	18.9 \pm 2.6 *	40 \pm 6
BSP	WT	0.5 \pm 0.1	47.4 \pm 4.4	102.2 \pm 8.8	100 \pm 9
	R57A	1.0 \pm 0.3 *	41.2 \pm 2.1	41.7 \pm 7.6 *	41 \pm 7
	R57K	0.4 \pm 0.0	42.7 \pm 4.8	106.0 \pm 8.8	104 \pm 9
	K361A	1.0 \pm 0.1 *	37.8 \pm 3.8	39.0 \pm 4.0 *	38 \pm 4
	K361R	1.0 \pm 0.2 *	28.1 \pm 1.3 *	27.7 \pm 2.5 *	27 \pm 2
estrone-3-sulfate	WT	0.4 \pm 0.0	74.7 \pm 8.3	198.5 \pm 14.0	100 \pm 7
	R57A	0.8 \pm 0.1 *	54.0 \pm 10.3	70.7 \pm 8.3 *	36 \pm 4
	R57K	0.7 \pm 0.1 *	49.6 \pm 3.1	71.0 \pm 7.7 *	36 \pm 4
	K361A	0.5 \pm 0.1 *	27.7 \pm 4.9 *	52.4 \pm 7.3 *	26 \pm 4
	K361R	1.8 \pm 0.3 *	56.9 \pm 5.6	31.0 \pm 3.7 *	16 \pm 2

Kinetic parameters of wild-type and mutated OATP1B1-mediated estradiol-17 β -glucuronide, BSP, and estrone-3-sulfate uptake were quantified under initial linear rate conditions and presented as mean \pm SE of 3-8 independent experiments. All V_{max} values are corrected for surface expression. Uptake of estradiol-17 β -glucuronide (1 μ M to 50 μ M) and BSP (0.05 μ M to 3 μ M) was quantified at 37°C for 1 min; uptake of estrone-3-sulfate (0.05 μ M to 2 μ M) was quantified at 37°C for 30 sec. Asterisks (*) indicate significant difference based on unpaired, two-tailed Student *t* test. *, *p*<0.05.

361 remained at about 30 to 40 %. The findings for BSP transport were very similar. Alanine replacement at both positions increased the apparent K_m values about two fold, whereas reintroduction of a positive charge only had an effect at position 57 (Table 3-1). The V_{max} values of the mutants did not differ significantly from wild-type, except for K361R for which the V_{max} value was reduced. When transport efficiencies were calculated, it became apparent that at position 57, alanine reduced the transport efficiency to 41 % of wild-type OATP1B1. Re-introduction of a positive charge at position 57 with a lysine increased the transport efficiency to the same as wild-type OATP1B1. In contrast, at position 361 the lysine is specifically required for proper transport because the alanine and the arginine mutants had V_{max}/K_m values of only 38 and 27 % of wild-type OATP1B1, respectively (Table 3-1), and re-introducing a positive charge did not improve transport efficiency. The alanine mutant R57A had a slightly decreased affinity for estrone-3-sulfate transport. The apparent K_m for R57A ($0.8\mu\text{M}$) was twice as high as for wild-type OATP1B1 ($0.4\mu\text{M}$). However, in contrast to estradiol-17 β -glucuronide and BSP, re-introduction of a positive charge with a lysine did not return the K_m value ($0.7\mu\text{M}$) significantly towards the value of wild-type OATP1B1. Because the V_{max} values only slightly decreased, the overall transport efficiencies were decreased for both mutants, mainly due to increased K_m values. The alanine mutation at position K361 did not affect the affinity for estrone-3-sulfate transport, but resulted in a 2.5 fold lower V_{max} value (Table 3-1). The mutation to an arginine decreased the affinity 4.5 fold from $0.4\mu\text{M}$ to $1.8\mu\text{M}$ with no significant change in V_{max} (Table 3-1). Thus the decreased

transport efficiencies were due to a decrease in the V_{\max} for K361A, and due to increase in the K_m for K361R. Taken together, these results strongly suggest that a positive charge at position 57 is important for estradiol-17 β -glucuronide and BSP transport, whereas a lysine at position 361 is absolutely required for transport of all three substrate tested. These conclusions are further supported by the fact that in all nine so far functionally characterized human OATPs a lysine is conserved at the position corresponding to 361 in OATP1B1, whereas a positive charge is conserved at the position corresponding to 57 in OATP1B1 (arginine at 57: OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP3A1, OATP4A1, and OATP5A1; lysine at 57: OATP2A1, OATP2B1, OATP4C1, and OATP6A1).

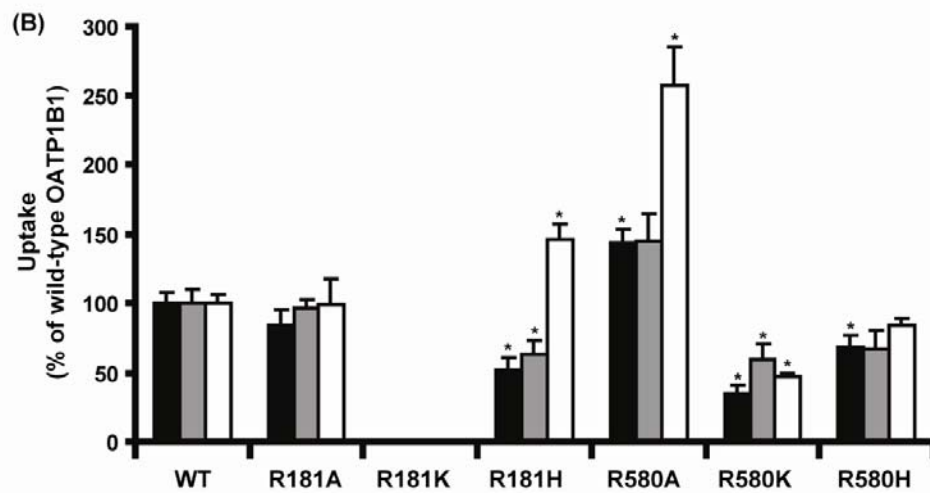
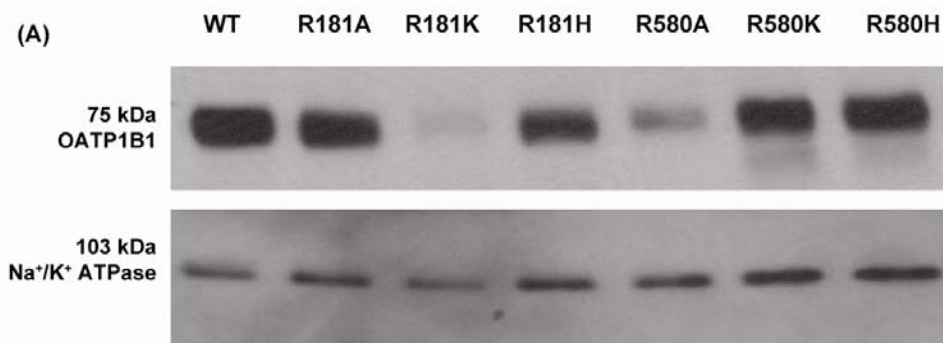
2.3 Expression and function of mutants in transmembrane domains.

To further study the roles of transmembrane R181 and R580 in transporting the different substrates we generated in addition to the alanine mutants also positively charged mutants R181K, R181H, R580K and R580H. As shown in Figure 3-5A, R181K was not expressed at the cell surface at all and therefore its function could not be determined whereas surface expression of R181H was reduced to about 60% of wild-type. Surface expression of R580A was reduced but surface expressions of R580K and R580H were normal. These data suggest that a positively charged amino acid at position 580 is essential for proper surface expression of the protein. Transport activities of all mutants for the three substrates, estradiol-17 β -glucuronide, estrone-3-sulfate, and BSP are compared in Figure 3-5B. Among the different transmembrane

Figure 3-5 Surface expression and transport function of transmembrane mutants R181 and R580.

(A) Representative western blots of surface-biotinylated proteins detected with a polyclonal anti-OATP1B1 antibody (75kDa). The same blot was also probed with an antibody against the plasma membrane marker Na^+/K^+ -ATPase α subunit (103 kDa) as a protein loading control.

(B) Uptake of $1\mu\text{M}$ [^3H]-estradiol- 17β -glucuronide (black bar), $0.1\mu\text{M}$ [^3H]-estrone-3-sulfate (gray bar) and $0.1\mu\text{M}$ [^3H]-BSP (white bar) was quantified individually in HEK293 cells expressing R181 or R580 mutant at 37°C under initial linear rate conditions and presented as % of wild-type after corrected for surface expression. R181K is not detectable on the cell surface, and thus the transport activity was too low to be quantified. Each bar is the mean \pm standard error (SE) of five independent experiments. Asterisk (*) indicate significantly different activities between wild-type and mutated transporters. *, $p < 0.05$.



mutants, R181A and R580H had almost normal transport activities for all of the tested substrates, whereas the R580K mutant had reduced transport activities. Interestingly, both R181H and R580A exhibited differences in transport of the three substrates. Mutant R181H had decreased estradiol-17 β -glucuronide transport, normal to slightly decreased estrone-3-sulfate transport, but increased BSP transport. Mutant R580A demonstrated moderately increased estradiol-17 β -glucuronide and estrone-3-sulfate (both to 140% of wild-type OATP1B1) uptake but strongly increased BSP (to 250% of wild-type OATP1B1) uptake. To investigate whether these differences in substrate handling of the transmembrane mutants were due to changes in apparent K_m or V_{max} , we performed kinetics analysis (Table 3-2). R181A showed no significant differences from wild-type for all three substrates tested. R181H had a slightly increased K_m for estradiol-17 β -glucuronide, whereas no significant changes in K_m were observed for estrone-3-sulfate and BSP. V_{max} values of R181H were not different from wild-type OATP1B1. Therefore, we conclude that a histidine at position 181 may affect substrate recognition for estradiol-17 β -glucuronide. However, we also conclude that the positive charge at this position is not directly involved in transport of the three substrates investigated, because it can be replaced by the neutral alanine without significant changes in transport activity. This conclusion is further supported by the fact that the R181 is only conserved within the OATP1 family.

Table 3-2: Kinetic parameters of transmembrane mutants

Substrate	Mutant	K _m (μ M)	V _{max} (pmol/mg protein per min)	V _{max} /K _m (μ l/mg protein per min)	% of WT
estradiol-17 β -glucuronide	WT	4.9 \pm 0.3	243.3 \pm 19.8	50.5 \pm 3.1	100
	R181A	6.6 \pm 0.2	213.2 \pm 10.8	32.5 \pm 0.8 *	60 \pm 7
	R181H	9.6 \pm 1.9 *	175.9 \pm 32.5	18.7 \pm 2.9 *	39 \pm 2
	R580A	7.9 \pm 0.5 *	575.6 \pm 27.2 *	73.5 \pm 5.7 *	135 \pm 11
	R580K	0.6 \pm 0.1 *	17.8 \pm 1.6 *	30.0 \pm 3.1 *	71 \pm 7
	R580H	2.0 \pm 0.1 *	77.1 \pm 3.9 *	38.3 \pm 0.9	91 \pm 2
estrone-3-sulfate	WT	0.4 \pm 0.0	65.1 \pm 9.5	189.5 \pm 30.2	100
	R181A	0.4 \pm 0.1	66.2 \pm 9.2	180.9 \pm 15.0	68 \pm 7
	R181H	0.4 \pm 0.1	44.7 \pm 11.9	107.9 \pm 13.6	61 \pm 11
	R580A	0.2 \pm 0.0 *	58.0 \pm 13.9	309.2 \pm 70.9 *	164 \pm 6
	R580K	0.2 \pm 0.0 *	19.9 \pm 3.9 *	120.6 \pm 15.9	67 \pm 7
	R580H	0.2 \pm 0.0 *	36.9 \pm 3.7 *	189.4 \pm 30.5	109 \pm 25
BSP	WT	0.5 \pm 0.1	47.3 \pm 4.8	101.8 \pm 4.5	100
	R181A	0.5 \pm 0.1	45.8 \pm 7.0	78.6 \pm 11.8 *	84 \pm 18
	R181H	0.3 \pm 0.1	39.4 \pm 8.0	160.2 \pm 19.5 *	150 \pm 22
	R580A	0.5 \pm 0.0	94.0 \pm 11.6 *	186.0 \pm 17.3 *	197 \pm 21
	R580K	1.2 \pm 0.1 *	31.5 \pm 1.6 *	27.5 \pm 2.6 *	28 \pm 3
	R580H	0.3 \pm 0.0	26.2 \pm 3.1 *	91.0 \pm 12.2	97 \pm 10

Kinetics experiments were done under the same conditions as described in Table 3-1.

*, $p < 0.05$.

At the other transmembrane position, mutant R580A showed increased transport efficiency for estradiol-17 β -glucuronide (145%), estrone-3-sulfate (167%), and BSP (189%) (Table 3-2). For estradiol-17 β -glucuronide and BSP, these effects were mainly due to a doubling of the V_{\max} values with a slight or no change in apparent K_m . However, for estrone-3-sulfate, the increase was mainly due to a decrease of the apparent K_m (from 0.4 to 0.2 μ M), whereas the V_{\max} remained the same. When we re-introduced a positive charge by a lysine at position 580, both K_m and V_{\max} values for all substrates were affected. For estradiol-17 β -glucuronide and estrone-3-sulfate, R580K had significantly decreased V_{\max} (estradiol-17 β -glucuronide from 243 to 18 pmol/mg per min; estrone-3-sulfate from 65 to 10 pmol/mg per min), and K_m values (estradiol-17 β -glucuronide from 4.9 to 0.6 μ M; estrone-3-sulfate from 0.4 to 0.2 μ M). For BSP, the V_{\max} value was slightly decreased (from 47 to 31 pmol/mg per min), whereas the K_m value was increased two fold (from 0.5 to 1.2 μ M) (Table 3-2). However, when we re-introduced a positive charge with a histidine at position 580, V_{\max} values for estradiol-17 β -glucuronide and estrone-3-sulfate increased as compared to the lysine mutation, but were still significantly lower than the wild-type. However, the V_{\max} value for BSP transport (26 pmol/mg/min) was about the same as for the lysine mutant R580K and slightly lower than wild-type OATP1B1, but the K_m value (0.3 μ M) was significantly lower (1.2 μ M). Compared to wild-type, the overall transport efficiencies of R580H for all three substrates were not altered due to simultaneous changes in V_{\max} and K_m values. The fact that both lysine and histidine mutations at 580 significantly decreased V_{\max} , suggests that arginine at this position is

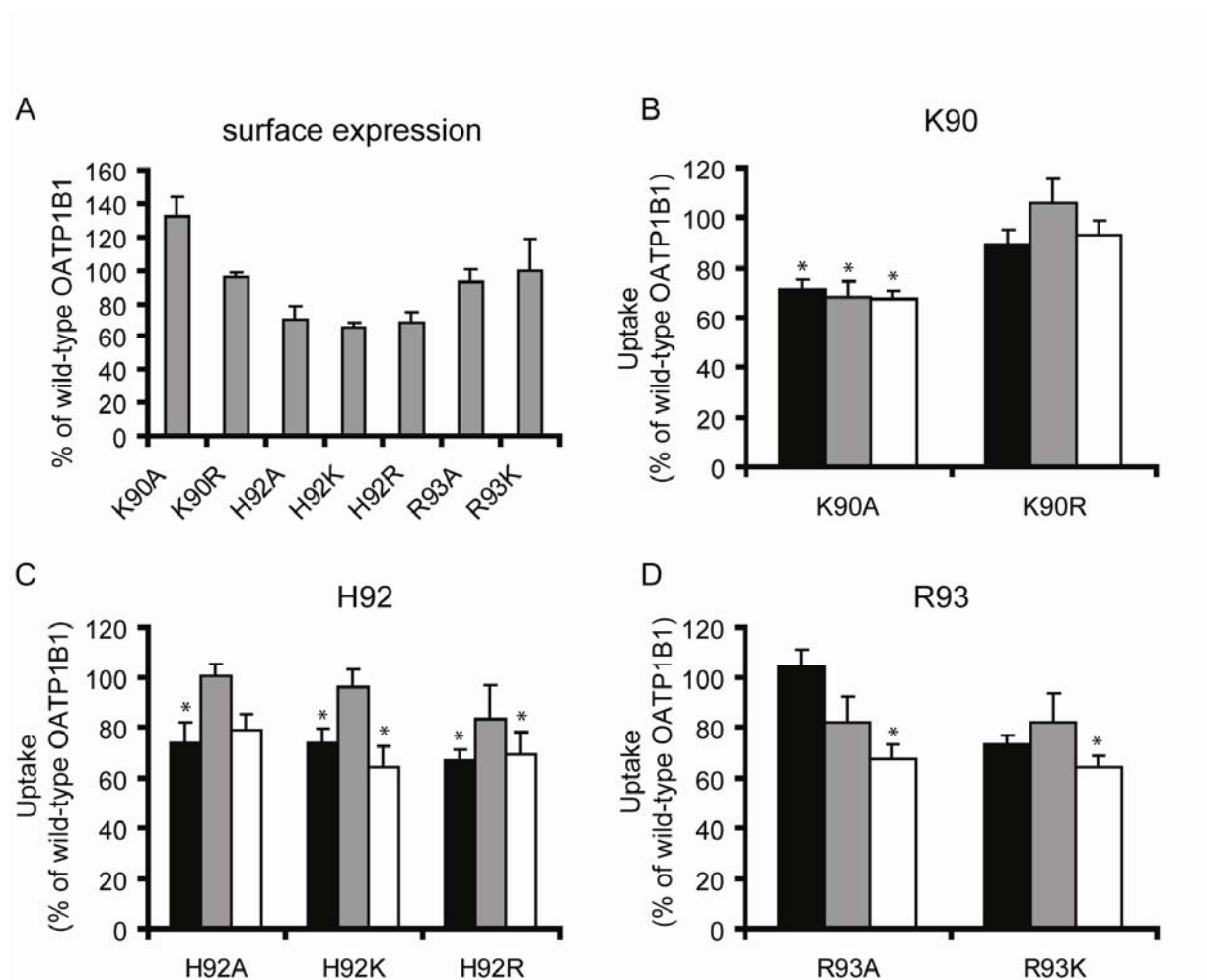
important for the translocation process. Furthermore, because both R580K and R580H mutations affected the turnover numbers for estradiol-17 β -glucuronide and estrone-3-sulfate more than that for BSP suggests that the different substrates may be handled differently by the transporter. Alternatively it is possible that interactions between other amino acid side chains and the different substrates are affected by the mutations. These results together with cell surface expression data suggest that R580 is involved in both maintaining the correct conformation of OATP1B1 at the cell surface and proper transport function. Such an important role for this amino acid position is also supported by an absolute conservation of the arginine residue at this position within all eleven human OATPs.

2.4 Expression and function of mutants on the intracellular side

Finally, we investigated the effects of mutations at the intracellular conserved positively charged residues. We constructed K90A, K90R, H92A, H92K, H92R, R93A, and R93K and determined their surface expression (Figure 3-6A), as well as uptake of estradiol-17 β -glucuronide (Figure 3-6B), estrone-3-sulfate (Figure 3-6C), and BSP (Figure 3-6D) mediated by these mutants. Except for the H92 mutants that had about 60% to 70% of wild-type surface expression (Figure 3-6A), all the other

Figure 3-6 Surface expression and transport function of intracellular K90, H92 and R93 mutants.

(A) Quantification of surface expression of intracellular mutants normalized to loading control Na^+/K^+ -ATPase presented as a percentage of wild-type OATP1B1 (WT). Each bar is the mean \pm standard error (SE) of 3-5 independent experiments. Uptake of $1\mu\text{M}$ [^3H]-estradiol- 17β -glucuronide (black bar), $0.1\mu\text{M}$ [^3H]-estrone-3-sulfate (gray bar), and $0.1\mu\text{M}$ [^3H]-BSP (white bar) was quantified individually in HEK293 cells expressing intracellular mutants K90 (B), H92 (C) and R93 (D) at 37°C under initial linear rate conditions, and is presented as % of wild-type after correction for surface expression. Each bar is mean \pm standard error (SE) of 5 independent experiments. *, $p < 0.05$.



mutants expressed normally on the cell surface. With respect to transport, K90A showed slightly decreased uptake for all three substrates (about 70% of wild-type), whereas K90R-mediated uptake was normal (Figure 3-6B). All three H92 mutants had slightly decreased estradiol-17 β -glucuronide and BSP uptake (65% to 80% of wild-type), but normal estrone-3-sulfate uptake (Figure 3-6C). R93 mutants showed decreased BSP uptake (about 65% of wild-type) but no significant effects on estradiol-17 β -glucuronide or estrone-3-sulfate transport (Figure 3-6D). These results suggest that a positively charged amino acid is required at position 90 for normal transport of all three substrates. However at positions 92 and 93, reintroducing positively charged amino acid residues did not restore normal transport activity of any of the substrates tested. Subsequently, we characterized the transport kinetics of the different mutants, and the results are summarized in Table 3-3. Apparent K_m values for all three substrates did not change for both K90A and K90R mutants, whereas K90A had slightly decreased V_{max} values. Therefore K90R had slightly higher overall transport efficiency values than K90A. All H92 and R93 mutants had only decreased V_{max} values for estradiol-17 β -glucuronide transport resulting in decreased overall transport efficiency. However, for estrone-3-sulfate and BSP transport, the overall V_{max}/K_m values for H92 and R93 mutants remained very close to the values for wild-type, due to simultaneously decreased K_m and V_{max} values.

In summary, a positively charged residue at position 90 seems to be preferred for normal translocation of all three substrates. At positions 92 and 93, the histidine

Table 3-3: Kinetic parameters of intracellular mutants

Substrate	Mutant	K _m (μ M)	V _{max} (pmol/mg protein per min)	V _{max} /K _m (μ l/mg protein per min)	% of WT
estradiol-17 β -glucuronide	WT	5.0 \pm 0.4	210.3 \pm 7.4	43.6 \pm 4.0	100
	K90A	4.8 \pm 1.0	162.2 \pm 15.5 *	35.1 \pm 3.9	74 \pm 3
	K90R	4.1 \pm 0.9	153.7 \pm 11.9 *	42.3 \pm 7.6	102 \pm 10
	H92A	4.3 \pm 0.7	108.3 \pm 15.4 *	25.9 \pm 3.9 *	65 \pm 11
	H92K	5.4 \pm 1.6	125.8 \pm 13.9 *	27.8 \pm 7.7	67 \pm 8
	H92R	4.9 \pm 0.1	114.6 \pm 7.8 *	23.5 \pm 1.0 *	61 \pm 7
	R93A	4.8 \pm 0.7	100.4 \pm 7.0 *	21.5 \pm 1.5 *	60 \pm 5
	R93K	4.6 \pm 1.2	108.9 \pm 5.6 *	27.8 \pm 8.2	67 \pm 10
estrone-3-sulfate	WT	0.4 \pm 0.0	74.7 \pm 8.3	199.6 \pm 21.7	100
	K90A	0.4 \pm 0.0	44.5 \pm 7.7	127.8 \pm 32.0	63 \pm 12
	K90R	0.3 \pm 0.0	63.1 \pm 9.1	193.4 \pm 15.1	124 \pm 9
	H92A	0.2 \pm 0.0 *	45.5 \pm 5.3	268.8 \pm 42.7	129 \pm 8
	H92K	0.1 \pm 0.0 *	36.3 \pm 10.0 *	280.2 \pm 32.2	160 \pm 25
	H92R	0.2 \pm 0.0 *	48.1 \pm 7.5	223.5 \pm 38.7	101 \pm 0
	R93A	0.2 \pm 0.0 *	41.6 \pm 11.7	219.3 \pm 41.5	107 \pm 5
	R93K	0.3 \pm 0.0 *	45.3 \pm 14.3	162.4 \pm 38.7	93 \pm 11
BSP	WT	0.4 \pm 0.1	48.6 \pm 4.9	121.1 \pm 7.1	100
	K90A	0.4 \pm 0.1	31.7 \pm 6.0	86.8 \pm 11.1 *	66 \pm 5
	K90R	0.4 \pm 0.1	46.5 \pm 7.5	133.7 \pm 9.0	104 \pm 4
	H92A	0.2 \pm 0.1 *	20.7 \pm 1.6 *	111.5 \pm 25.3	104 \pm 29
	H92K	0.2 \pm 0.1 *	15.2 \pm 1.6 *	99.6 \pm 27.7	87 \pm 17
	H92R	0.2 \pm 0.0 *	14.7 \pm 3.7 *	94.7 \pm 2.3	88 \pm 10
	R93A	0.2 \pm 0.0 *	22.6 \pm 0.8 *	132.7 \pm 11.6	104 \pm 3
	R93K	0.2 \pm 0.0 *	18.4 \pm 1.4 *	100.2 \pm 11.3	90 \pm 0

Kinetics experiments were done under the same conditions as described in Table 2. *, $p < 0.05$.

and arginine, rather than just the positive charge, seem to affect transport activity. These findings are also consistent with the fact that H92 is conserved throughout the human OATP1 to OATP4 subfamily, and R93 is conserved throughout the OATP1 to OATP3 subfamily, whereas K90 is only conserved in the OATP1 family and an arginine can be found at this position for almost all the other OATPs. However, all three intracellular residues (K90, H92 and R93) are not as important for transport function compared to the two extracellular residues (R57 and K361) and one transmembrane domain residue (R580).

In conclusion, for proteins with unknown structure and transport mechanisms, comparative modeling combined with the analysis of conserved amino acid residues is a powerful tool to identify candidate residues that may play important roles in structure and/or function of a transporter. Site-directed mutagenesis has provided useful structural and mechanistic information for the bacterial transporters in the major facilitator superfamily, lactose permease (Abramson et al., 2003), glycerol-3-phosphate transporter (Huang et al., 2003) and multidrug transporter EmrD (Yin et al., 2006) prior to their crystallization. Because of the mainly anionic nature of OATP1B1 substrates, we used site-directed mutagenesis to investigate the roles of several conserved positively charged amino acids facing the putative binding pocket of OATP1B1. We have shown the important role of the positive charge at R57 for substrate recognition/binding and at R580 for surface expression. We also demonstrated that two conserved amino acids are essential, K361 for substrate

binding/translocation, and R580 for substrate translocation. These findings are important steps towards elucidating the structure-function relationship of OATP1B1.

CHAPTER 4

IDENTIFICATION OF AMINO ACID RESIDUES ESSENTIAL FOR EFFICIENT TRANSPORT MEDIATED BY RAT Oatp1a4

SPECIFIC AIM AND HYPOTHESIS

Chapter 4 will address specific aim 2 and will evaluate the hypothesis that **quantifying transport activities of different substrates mediated by chimeras between rat Oatp1a1 and Oatp1a4 in combination with site-directed mutagenesis should allow us to identify regions and/or individual amino acids that are important for Oatp1a4-mediated substrate recognition and/or transport.** The purpose of this study is to **determine the critical region and/or residues that are important in Oatp1a4-mediated transport and to further characterize the roles of these amino acids in the transport processes.**

I. INTRODUCTION

Organic anion transporting polypeptides (rodents: Oatps; human: OATPs) form a superfamily of membrane transport proteins that are subdivided into 6 mammalian families (≥ 40 % amino acid sequence identities, OATP1-6) and 13 subfamilies (≥ 60 % amino acid sequence identities) (Hagenbuch and Meier, 2004). The OATP1 family is the best characterized family and contains the rodent Oatp1a1 (gene symbol *Slco1a1*), Oatp1a3 (*Slco1a3*), Oatp1a4 (*Slco1a4*), Oatp1a5, (*Slco1a5*), Oatp1a6 (*Slco1a6*), Oatp1b2 (*Slco1b2*), Oatp1c1 (*Slco1c1*) as well as the human OATP1A2

(*SLCO1A2*), OATP1B1 (*SLCO1B1*), OATP1B3 (*SLCO1B3*) and OATP1C1 (*SLCO1C1*). Most of these OATP/Oatps are multispecific carriers that transport a variety of amphipathic organic compounds, including bile acids, organic anionic dyes, steroid conjugates, thyroid hormones, prostaglandins, anionic oligopeptides, numerous drugs, and other xenobiotics (Hagenbuch and Meier, 2003). All OATPs have been predicted to have 12-transmembrane domains (TM) and the 12-TM topology of Oatp1a1 has been confirmed experimentally using epitope-specific antibodies and immunofluorescence (Wang et al., 2008).

A few studies have been reported investigating the molecular basis of the substrate specificity of members of the OATP-gene superfamily and several structural elements involved in substrate binding and/or translocation have been identified. Experiments with the OATP2A1/Oatp2a1 from human, rat, and mouse, as well as some of their chimeras suggest that the C-terminal half of the protein is important for the apparent affinity of prostaglandin E₂ (Pucci et al., 1999). Using cysteine-scanning mutagenesis, the same group mapped the substrate-binding site of the rat Oatp2a1 to TM 10 (Chan et al., 1999) and identified conserved cationic amino acids important for prostaglandin transport (Chan et al., 2002). Furthermore, using chimeric proteins between OATP1B1 and OATP1B3 in combination with site-directed mutagenesis, several amino acids in TM10 of OATP1B3 have been identified to be important for CCK-8 transport (Gui and Hagenbuch, 2008). Similar strategies have been employed for OATP1B1, in which TM9 and TM8 (Miyagawa et al., 2009) or TM10 (Gui and

Hagenbuch, 2009) have been identified to affect the transport of estrone-3-sulfate and/or estradiol-17 β -glucuronide. However, no such studies have been performed for transporters in the OATP1A/Oatp1a subfamily.

Among the five rat transporters in the Oatp1a subfamily, only Oatp1a1 (Jacquemin et al., 1994) and Oatp1a4 (Noe et al., 1997) have been shown to be expressed in the liver. Besides common substrates like the bile acid taurocholate (Noe et al., 1997; Eckhardt et al., 1999), the steroid-conjugate estradiol-17 β -glucuronide (Kanai et al., 1996; Noe et al., 1997; Eckhardt et al., 1999) and the opioid peptide D-penicillamine^{2,5} enkephalin (DPDPE) (Noe et al., 1997; Kakyo et al., 1999; Reichel et al., 1999; Gao et al., 2000), there are compounds that are specific substrates for either Oatp1a1 or Oatp1a4. For example, digoxin is a good substrate for Oatp1a4 but is not transported by Oatp1a1 (Cattori et al., 2001). Bromosulfophthalein (BSP) in contrast, has been shown to be transported by Oatp1a1 but not by Oatp1a4 (Gao et al., 2000).

Based on these substrate specificities and the overall high amino acid sequence identity of 77%, we hypothesized that constructing chimeric proteins between Oatp1a1 and Oatp1a4 in combination with site-directed mutagenesis, and quantifying transport uptake of the Oatp1a4-specific digoxin would allow us to identify regions that are important for Oatp1a4-mediated substrate transport.

II. RESULTS AND DISCUSSION

2.1 Digoxin transport with chimeras between Oatp1a1 and Oatp1a4.

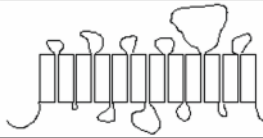


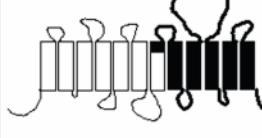

In order to examine the structural requirements for Oatp1a4-mediated transport, we constructed chimeras between Oatp1a1, which does not transport digoxin, and Oatp1a4, which represents a high-affinity digoxin transporter, and quantified uptake of digoxin. Using digoxin transport as a functional tool should allow us to identify regions that are important for Oatp1a4-mediated digoxin transport.

Chimeras were constructed using an overlap PCR approach with primers that were identical for Oatp1a1 and Oatp1a4. Because the nucleotide sequence identity between Oatp1a1 and Oatp1a4 determined the location of the primers (Tables 2-3 and 2-4), chimeras did not necessarily begin or end at the beginning or end of a certain TM (Figure 4-1A). Chimera 1 contained TM 1-7 of Oatp1a1 followed by the C-terminal TM 8-12 of Oatp1a4, and chimera 2 was constructed as the mirror image of chimera 1 containing TM 1-7 of Oatp1a4 followed by TM 8-12 of Oatp1a1. In order to be able to detect all chimeras as well as Oatp1a1 and Oatp1a4 with the same antibody, all constructs were made with a C-terminal His-tag. Together with His-tagged wild-type Oatp1a1 and Oatp1a4, chimeras 1 and 2 were expressed in *X. laevis* oocytes. As published previously (Noe et al., 1997), Oatp1a4 mediated digoxin transport had a more than 10-fold signal to background ratio in this expression system. Furthermore, preliminary experiments revealed that the His-tagged Oatps transported typical substrates at the same rates as the wild-type Oatps (data not

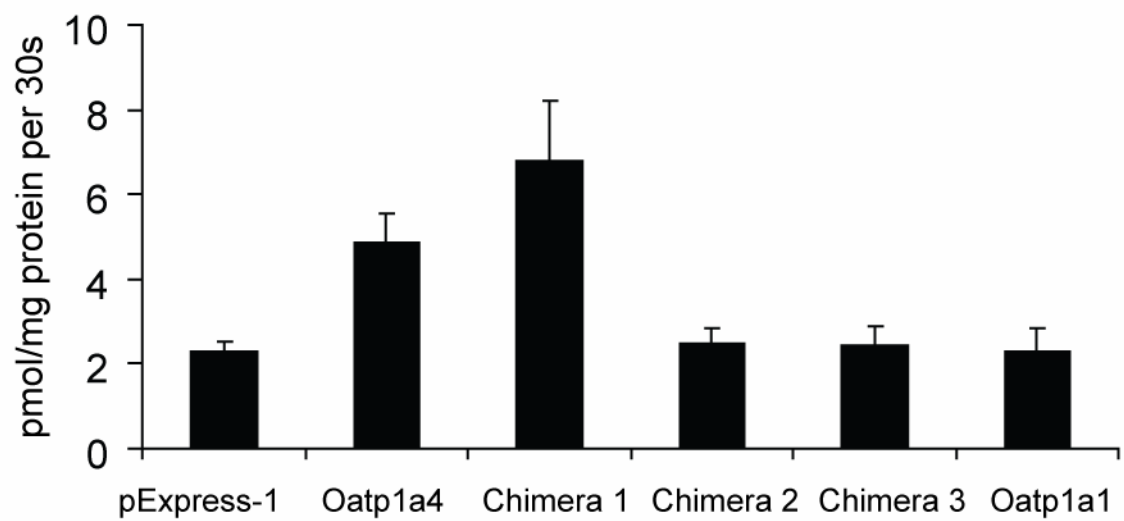
Figure 4-1: Digoxin transport by wild-type and chimeric Oatp1a1 and Oatp1a4 constructs in oocytes (A) and HEK293 cells (B).

(A) *X. laevis* oocytes were injected with 50 nl water or 5 ng cRNA of the respective His-tagged constructs and after three days in culture, transport of 0.5 μ M [3 H]-digoxin was determined at 25 °C with 9-12 oocytes per condition. Black boxes and thick lines represent Oatp1a1, whereas white boxes and thin lines represent Oatp1a4. Values are given as percent of the initial transport rates obtained for wild-type Oatp1a4-injected oocytes and correspond to the mean of three (one for chimera 3) independent experiments. (B) Uptake of 0.5 μ M [3 H]-digoxin was quantified at 37°C for 30 sec (initial linear rate condition) with empty vector pExpress-1, Oatp1a4, Oatp1a1 or three chimeric transporter expressing HEK293 cells 48 hrs after transfection. Each bar is mean \pm standard deviation of triplicates.

A

Construct	Digoxin Transport	Construct	Digoxin Transport
 Oatp1a4 His	100 %	 Oatp1a1 His	7 %
 Chimera 1 His	88 %	 Chimera 2 His	6 %
 Chimera 3 His	3 %		

B



shown). Initial uptake rates for digoxin were determined and expressed in percent of the uptake obtained for wild-type Oatp1a4-expressing oocytes. Compared to Oatp1a4, only chimera 1 was able to mediate significant transport of digoxin, whereas chimera 2 and Oatp1a1 did not transport digoxin (Figure 4-1A). Because this construct contained the C-terminal TM 8-12 of Oatp1a4 and the reciprocal chimera 2 did not mediate digoxin transport, we concluded that TM 8-12 are important for Oatp1a4-mediated digoxin transport. To further investigate how much of this C-terminal portion of Oatp1a4 is needed for digoxin transport, an additional chimera was produced (chimera 3) and functionally tested in oocytes. The intracellular loop 4 has the same amino acid sequence in both Oatps. Therefore chimera 3 differs from chimera 1 only with respect to TM8 and extracellular loop (EL) 4. Because chimera 3 did not transport digoxin (Figure 4-1A), we concluded that the amino acid residues in TM8 and EL4, and/or their interactions with the rest of the C-terminal half of Oatp1a4 are important for Oatp1a4 mediated digoxin transport.

In order to test whether these results were expression system dependent, we transiently expressed these His-tagged chimeric proteins along with wild-type Oatp1a4 and Oatp1a1 in HEK293 cells and quantified digoxin transport uptake. Figure 4-1B shows the transport activities of the three chimeric transporters observed in HEK293 cells. Chimera 1 was able to transport digoxin but chimeras 2 and 3 were not. Thus, in both expression systems, amino acid residues in TM8 and EL4 or their

interactions with the rest of the C-terminal half of Oatp1a4 are important for digoxin transport.

2.2 Surface expression of chimeras between Oatp1a1 and Oatp1a4 in HEK293 cells.

In order to test whether the reduced digoxin transport activity of chimeras 2 and 3 was due to reduced surface expression levels, we examined surface expression of Oatp1a4, Oatp1a1, and the three chimeric proteins in HEK 293 cells using surface biotinylation experiments. As shown in Figure 4-2A, all three chimeric transporters were detected on the cell surface. However, the surface expression level of chimera 3 was about 50% of chimeras 1 and 2. Thus, the amino acids that differ between chimera 1 and 3 seem to be important for proper surface expression and transport. Two major bands, one at about 60 kDa and the other at about 85 kDa, were detected for chimera 1 and Oatp1a1. For Oatp1a4 and chimera 2 only one band close to the higher molecular weight, and for chimera 3 only one major band close to the lower molecular weight were seen (Figure 4-2A). This surface expression pattern was consistent throughout various experiments. Na^+/K^+ ATPase, a membrane protein naturally expressed in HEK293 cells was used as a loading control (Figure 4-2B).

After PNGase F treatment of purified surface proteins, only one major band with a decreased apparent molecular weight was detected for all constructs (Figure 4-2C), indicating that the multiple bands of chimera 1, chimera 3 and Oatp1a1 are due

to different N-glycosylation states. It has been shown in HeLa cells that of the four N-glycosylation sites, the three at positions N124, N135 and N492 are used in Oatp1a1 (Wang et al., 2008). The first two sites N124 and N135 are located in the N-terminal half of Oatp1a1, and the third one (N493) in the C-terminal half. Therefore, for Oatp1a1 and chimera 1, the lower band probably corresponds to a partially glycosylated form, and the upper band was the fully glycosylated form. Chimera 3, in which TM8 and EL4 were replaced with Oatp1a1 sequences, is probably only partially glycosylated. This could be due to a conformation change of the chimeric protein compared to the wild-type protein that decreases the accessibility of the N-glycosylation sites to the glycosylation enzymes and/or substrates. For Oatp1a4, although not confirmed experimentally, it has been predicted that in the N-terminal half, in addition to N123 and N134, which are equivalent to N124 and N135 in Oatp1a1, there is another N-glycosylation site N140. This additional site in Oatp1a4 might increase the chance for full N-glycosylation, because all three sites (N124, N135 and N140) are very close to each other. Therefore, only one major glycosylated band was observed for Oatp1a4 and Chimera 2, which shares the N-terminal half of wild-type Oatp1a4.

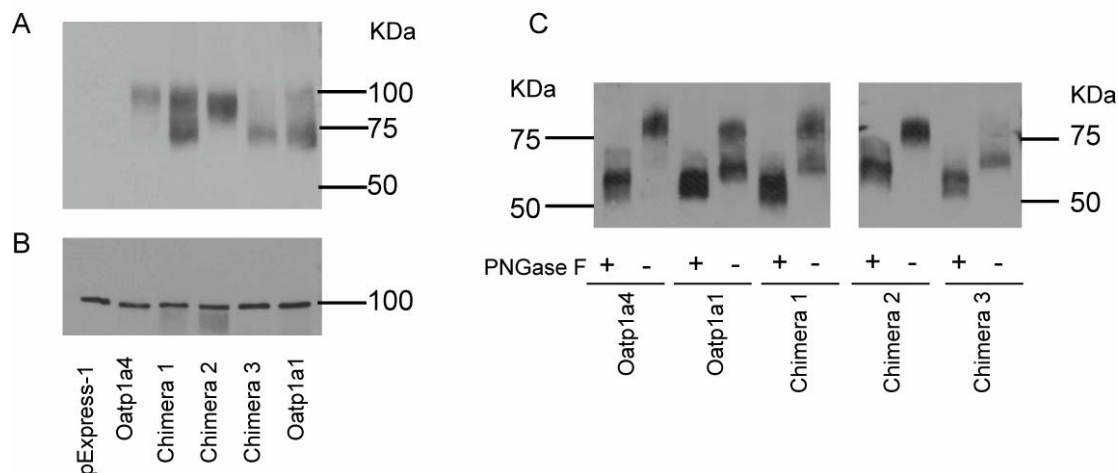


Figure 4-2: Surface expression of chimeric Oatp1a1 and Oatp1a4 constructs in HEK293 cells.

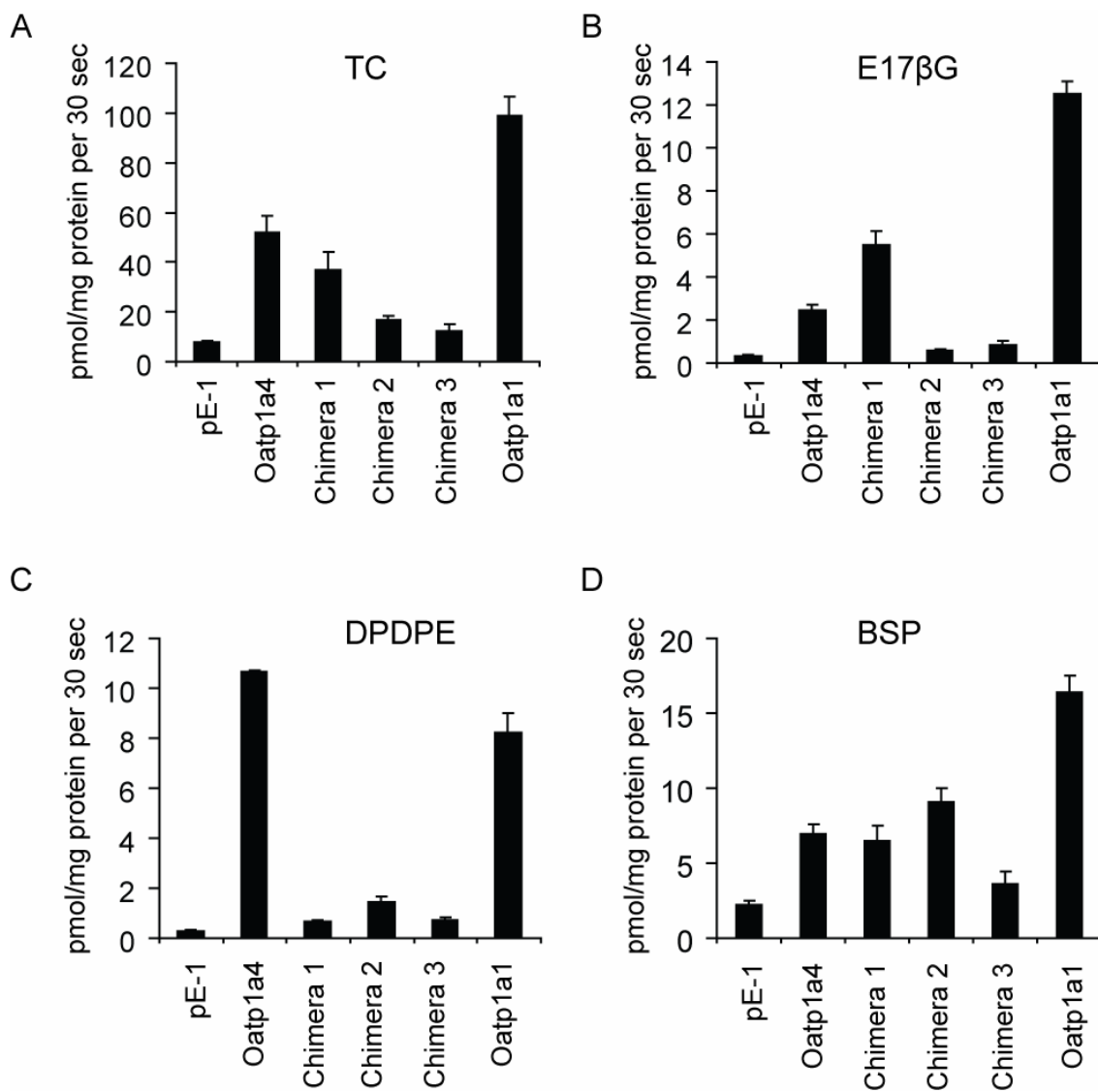
(A) Representative Western blots of surface-biotinylated proteins detected with a monoclonal anti-tetra-His antibody. HEK293 cells transfected with empty vector pExpress-1 were used as negative controls. (B) The same blot was also probed with an antibody against the plasma membrane marker Na⁺/K⁺-ATPase α subunit as a loading control. (C) Surface-biotinylated proteins were incubated with N-Glycosidase F (PNGase F) for 1 hr at 37°C for deglycosylation and detected with anti-tetra-His antibody.

2.3 Taurocholate, estradiol-17 β -glucuronide, DPDPE and BSP transport by chimeras between Oatp1a1 and Oatp1a4 in HEK293 cells.

Given the substrate multi-specificity of Oatps, we extended the studies beyond digoxin transport and tested the four general Oatp substrates taurocholate, estradiol-17 β -glucuronide, DPDPE, and BSP. In HEK293 cells expressing Oatp1a1 and Oatp1a4, taurocholate and estradiol-17 β -glucuronide are good substrates for both Oatp1s (Figure 4-3A and B). Similar to digoxin, chimera 1 was able to transport taurocholate (Figure 4-3A) and estradiol-17 β -glucuronide (Figure 4-3B) better than Chimera 2 and Chimera 3, suggesting that these three substrates may be handled similarly. However, although DPDPE was transported well in both Oatp1a4 and Oatp1a1 expressing cells as compared to the vector control cells, chimera 1 and chimera 3 did not mediate any transport, and the uptake mediated by chimera 2 was only 10% of Oatp1a4 and 14% of Oatp1a1 (Figure 4-3C). It has been shown previously in the *X. laevis* system that BSP is a substrate for Oatp1a1 but not for Oatp1a4 (Reichel et al., 1999). However, when we expressed Oatp1a4 in HEK293 cells we could measure BSP transport that was about twice the value obtained with the empty vector (Figure 4-3D). Oatp1a1-mediated BSP transport was only 6 times the value of the empty vector control (Figure 4-3D). Therefore, we conclude that BSP is a substrate of Oatp1a4 when expressed in HEK293 cells. These results can be explained by the fact that the experiments in oocytes were performed at a BSP concentration of 4 μ M as compared to 0.5 μ M in the HEK293 cell system. Furthermore, the signal obtained for Oatp1a1 in oocytes was 25 times above

Figure 4-3: Transport of taurocholate (A), estradiol-17 β -glucuronide (B), DPDPE (C) and BSP (D) mediated by wild-type and chimeric Oatp1a1 and Oatp1a4 constructs in HEK293 cells.

Uptake of 20 μ M [3 H]-taurocholate (A), 0.5 μ M estradiol-17 β -glucuronide (B), 1 μ M DPDPE (C) and 0.5 μ M BSP (D) was quantified at 37°C for 30 sec (initial linear rate condition) with HEK293 cells expressing empty vector pExpress-1 (pE-1), Oatp1a4, Oatp1a1 or the three chimeric transporters 48 hrs after transfection. Each bar represents the mean \pm standard deviation of triplicate determinations.



background whereas in HEK293 cells it was only 6 times above empty vector cells. Thus, a twofold signal with overlapping standard deviations in oocytes compared to the 25-fold signal for Oatp1a1 probably led the authors to conclude that Oatp1a4 does not transport BSP. Both Chimera 1 and Chimera 2 were able to transport BSP whereas chimera 3 did not transport BSP (Figure 4-3D).

The same chimeric protein transported different substrates differently. Chimera 1 was able to transport taurocholate, estradiol-17 β -glucuronide, and BSP, but not DPDPE, which indicates that Oatp1a4 is similar to other OATP/Oatps (Tamai et al., 2001; Noe et al., 2007) in that it has multiple binding sites for various substrates. Unlike for most of the Oatp1a4 substrates tested, the presence of only the C-terminal half of Oatp1a4 was not adequate for DPDPE transport, neither was the presence of only the N-terminal half. It is therefore likely that the transport pathway for DPDPE requires interactions between the two halves, whereas for other Oatp1a4 substrates, critical interactions are primarily in the C-terminal region. Alternatively, interactions with amino acids of the Oatp1a1 part may result in a functional protein for substrates other than DPDPE. The concept of multiple binding sites is also consistent with the kinetic and inhibition studies reported earlier (Sugiyama et al., 2002). Those data suggested that estradiol-17- β -glucuronide could interact at a different recognition site than taurocholate and digoxin. In the current study, the C-terminal half of Oatp1a4 seems to be important for the transport of digoxin, estradiol-17- β -glucuronide, and taurocholate.

Whereas chimera 1, which has the N-terminal half of Oatp1a1 and the C-terminal half of Oatp1a4, was able to transport the two common substrates taurocholate and estradiol-17 β -glucuronide, its reciprocal chimera 2, which has the C-terminal half of Oatp1a1, did not transport the two substrates. However, both chimera 1 and chimera 2 were able to transport BSP. These results suggest that BSP may be transported using a similar pathway in both Oatp1a1 and Oatp1a4, whereas for other common substrates, the translocation pathway requires different amino acids that cannot be complemented by the other Oatp.

Finally, chimera 3 was not able to transport any of the 5 substrates tested. However, chimera 1 with TM8 and EL4 added to the rest of the C-terminal part of Oatp1a4, was able to transport all substrates except for DPDPE. Therefore, it is possible that TM8 and EL4 are required for maintaining the correct conformation of the binding/translocation pathway. This result is consistent with a recent study that reported that when replacing TM8 of OATP1B1 with OATP1B3, transport rates for estradiol-17 β -glucuronide and estrone-3-sulfate were markedly decreased (Miyagawa et al., 2009). Because a reduced surface expression with only partial glycosylation of chimera 3 was observed on western blots, the lack of full glycosylation and (as a consequence the lack of) surface expression could also contribute to the decreased transport rates. However, to understand whether the N-glycosylation will affect transport function, more studies are needed.

Given that digoxin, taurocholate, and estradiol-17 β -glucuronide were handled in a very similar way, we decided to continue with digoxin as a functional tool and compare the amino acid sequence of the region that differs between chimera 1 and 3.

2.4 Comparison of amino acids 324 to 387 of Oatp1a4 with Oatp1a1 and Oatp1a5.

In order to identify the role of single amino acids in Oatp1a4-mediated digoxin transport, the 64 amino acids of Oatp1a4 that differ between chimera 1 and chimera 3 were aligned to the corresponding amino acids of Oatp1a1 and Oatp1a5 (Figure 4-4A). The multiple alignments of both Oatp1a4 and Oatp1a5 with Oatp1a1 revealed that there are only 6 amino acid residues that differ in the digoxin transporters, Oatp1a4 and Oatp1a5, compared with Oatp1a1, the non-digoxin transporter (Figure 4-4A, boxed). F328 of Oatp1a4 corresponds to a valine in Oatp1a1, A330 to a glycine, S334 to a positively charged lysine, V353 to an alanine, V354 to an isoleucine and M361 to a serine. As shown in Figure 4-4B, these six amino acid residues are all located close to the extracellular border of TM 7 and 8 or within TM7 and TM8 of the predicted membrane topology of Oatp1a4.

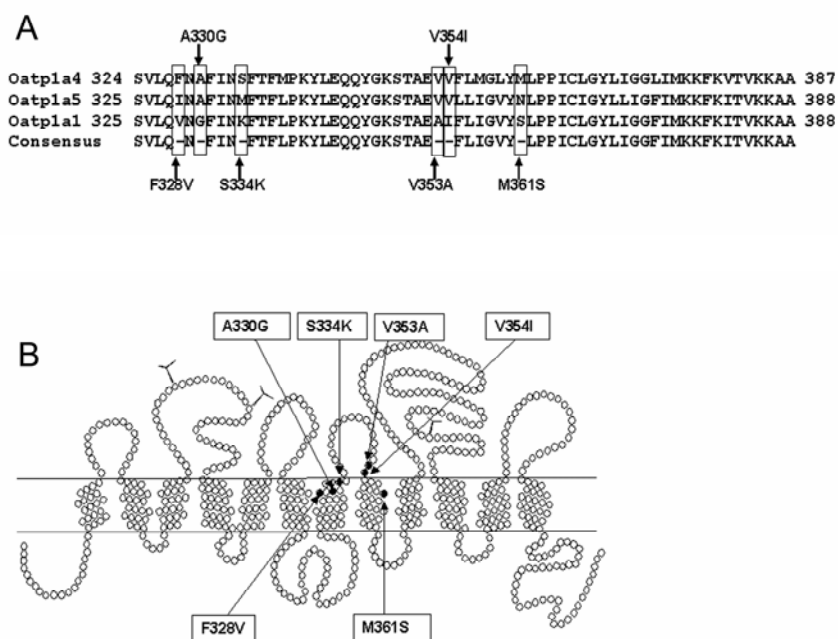


Figure 4-4: Partial amino acid sequence alignment and location of mutations in rat Oatp1a4 around TM 7 and 8.

(A) The 64 amino acids that were replaced from chimera 1 to chimera 3 (Figure 4-1) are shown and the amino acid residues that differ between Oatp1a1 and both Oatp1a4 and Oatp1a5 are boxed and labeled. (B) Location of the six amino acid residues is shown in the predicted membrane topology of Oatp1a4.

2.5 Site-directed mutagenesis of Oatp1a4 and functional characterization of the mutants in *X. laevis* oocytes.

Because any of the identified amino acid residues could be important for Oatp1a4-mediated digoxin transport, each of the six residues was changed individually in His-tagged Oatp1a4 to its counterpart amino acid residue in Oatp1a1. Because *X. laevis* oocyte system provides a very sensitive signal to background ratio, the resulting mutant Oatp1a4 proteins were first expressed in oocytes and digoxin transport rate was compared to wild-type Oatp1a4. As shown in Figure 4-5, digoxin transport rates of mutants A330G, V353A, and V354I were comparable to wild-type Oatp1a4. In contrast, digoxin transport rates obtained with mutant F328V was about 60% of wild-type Oatp1a4 with high variations and S334K as well as M361S were less than 40%. These results suggest that in the *X. laevis* oocyte system, the two individual Oatp1a4 mutants S334 and M361 are important for Oatp1a4 mediated digoxin transport and F328 might be important for Oatp1a4 mediated digoxin transport.

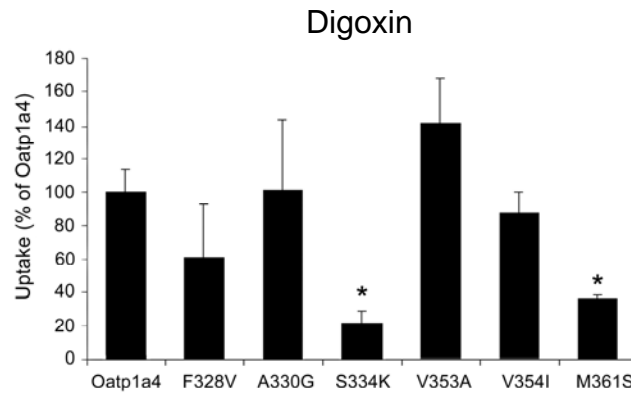


Figure 4-5: Digoxin transport by wild-type and mutated Oatp1a4 in oocytes.

X. laevis oocytes were injected with 50 nl water or 5 ng cRNA of the respective constructs and after three days in culture transport of 0.5 μ M [3 H]-digoxin was determined at 25 °C with 9-12 oocytes per condition. Values represent the means \pm S.E. of three independent experiments and are given as percent of the initial transport rates obtained for wild-type Oatp1a4-injected oocytes. * $p < 0.05$ compared to Oatp1a4.

2.6 Expression and functional analysis of Oatp1a4 mutants F328V, S334K, and M361S in HEK293 cells

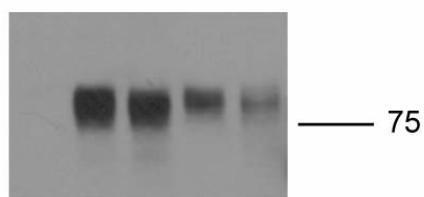
In order to confirm the results in oocytes and measure the surface expression of the mutants, we transiently expressed Oatp1a4 and mutants F328V, S334K, and M361S in HEK293 cells. Membrane proteins were purified using surface biotinylation, and western blot analysis was performed using the anti-His antibody. As demonstrated in Figure 4-6A, all Oatp1a4 constructs could be detected on the cell surface at different expression levels. Compared to wild-type Oatp1a4, F328V had the same surface expression level whereas the cell surface expression level of S334K and M361S was decreased. Na⁺/K⁺ ATPase was used as a loading control (Figure 4-6B). Quantification of several blots from different experiments have shown that on average, the surface expression level of S334K was about 50% and the surface expression level of M361S was less than 20% of wild-type Oatp1a4. The reason for the decreased surface expression level of these two mutants could be that either the original amino acids are critical for maintaining a stable structure, or the introduction of a new amino acid destabilizes the structure of the transporter.

Digoxin transport was also quantified in HEK293 cells transiently transfected with Oatp1a4 and the three mutants (Figure 4-6C). Digoxin transport mediated by F328V was the same as that by wild-type Oatp1a4, whereas S334K and M361S mediated digoxin transport was less than 20% of wild-type. The results for S334K

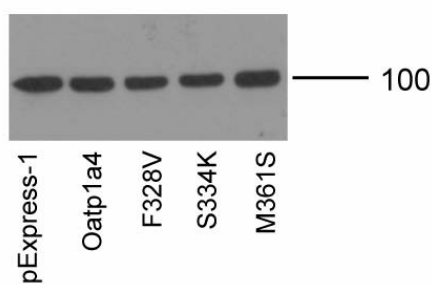
Figure 4-6: Surface expression and digoxin transport of wild-type and mutated Oatp1a4 constructs in HEK293 cells.

(A) Representative Western blots of surface-biotinylated proteins detected with a polyclonal anti-6-His antibody. HEK293 cells transfected with empty vector pExpress-1 were used as negative controls. (B) The same blot was also probed with an antibody against the plasma membrane marker Na⁺/K⁺-ATPase α subunit as a loading control. (C) Uptake of 0.5 μ M [³H]-digoxin was quantified at 37°C for 30 sec (initial linear rate condition) with HEK293 cells expressing empty vector pExpress-1, Oatp1a4 or a mutated transporter. The net uptakes were obtained by subtracting the uptake of empty vector transfected cells from the uptake of Oatp1a4 or mutant transfected cells. The results were normalized for total protein expression and presented as percentage of wild-type Oatp1a4. Each bar represents the mean \pm standard deviation of three independent experiments with triplicates. (D) Uptake of increasing concentrations of digoxin was quantified under initial linear rate conditions (30 sec) at 37°C with HEK293 cells expressing empty vector, wild-type Oatp1a4 or F328V. After subtracting the values obtained with the empty vector transfected HEK293 cells and corrected for total protein concentration, net uptake was fitted to the Michaelis-Menten equation. Each point represents the mean \pm standard deviation of triplicate determinations.

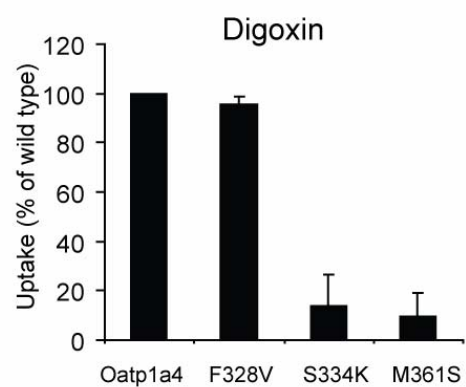
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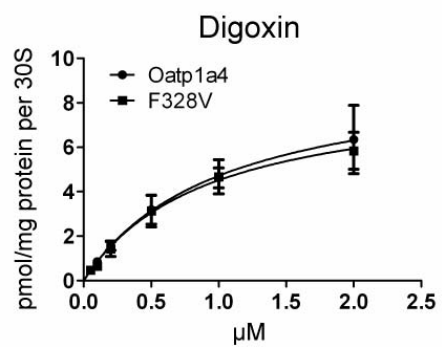
B



C



D



and M361S were consistent with the observations in the *X. laevis* oocyte system. Because M361S was minimally expressed on the cell surface, the reduced transport rate is probably mainly due to the lack of surface expression. For S334K however, where the surface expression level on average is about half of wild-type Oatp1a4 but the observed transport rate is less than 20% of wild-type Oatp1a4, it means that this mutation probably affects both surface expression and transport activity. Although in the *X. laevis* oocyte system, F328V had a slightly decreased transport rate, this mutation did not affect the digoxin transport rate in HEK293 cells under initial linear rate conditions. The concentration dependent uptake is shown in Figure 4-6D and further demonstrates that at low concentrations, F328V had the same transport kinetics as wild-type Oatp1a4. Therefore, in HEK293 cells, the F328V mutation does not affect either surface expression or transport of digoxin, whereas the S334K mutation may affect both surface expression and transport rate. M361S mainly affected surface expression.

2.7 Taurocholate, estradiol-17 β -glucuronide, DPDPE, and BSP transport by wild-type and mutated Oatp1a4 in HEK293 cells.

The transport rates of the four substrates mediated by the three mutants F328V, S334K, and M361S were also quantified in HEK293 cells. As shown in Figure 4-7, uptake of taurocholate, estradiol-17 β -glucuronide, and DPDPE mediated by F328V

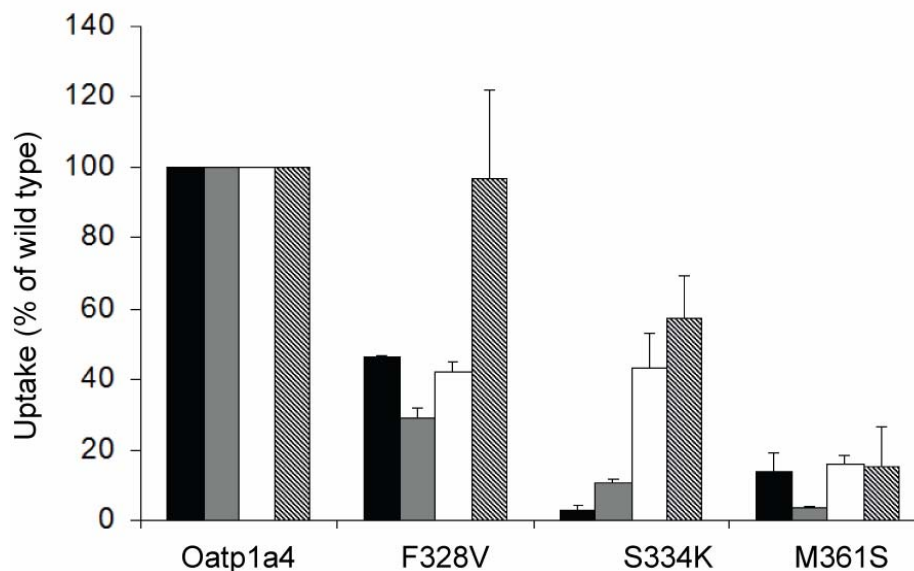


Figure 4-7: Transport of taurocholate, estradiol-17β-glucuronide, DPDPE, and BSP mediated by wild-type and mutated Oatp1a4 in HEK293 cells.

Uptake of 20μM [³H]-taurocholate (black), 0.5 μM estradiol-17β-glucuronide (gray), 1 μM DPDPE (white) and 0.5 μM BSP (hatched) was quantified at 37°C for 30 sec (initial linear rate condition) with HEK293 cells expressing empty vector pExpress-1, Oatp1a4, or the mutated transporter. The net uptakes were obtained by subtracting the uptake of empty vector transfected cells from the uptake of Oatp1a4 or mutant transfected cells. The results were normalized for total protein expression and presented as percentage of wild-type Oatp1a4. Each bar is mean ± standard deviation of three independent experiments with triplicate determinations.

was decreased to 40% - 50% of the wild-type Oatp1a4 whereas BSP uptake, similar to digoxin, remained the same. For S334K, uptake of taurocholate and estradiol-17 β -glucuronide was less than 20% of the wild-type, whereas DPDPE and BSP uptake was about 50% - 60% of the wild-type. The transport rates of all four common substrates mediated by M361S were less than 20% of the wild-type Oatp1a4.

The mutation F328V had different effects on different substrates. It did not affect transport rates of digoxin or BSP, but the transport rates of taurocholate, estradiol-17 β -glucuronide and DPDPE were decreased. This can be explained in different ways. 1) Valine interacts with some of the substrates and thus slows their transport process; or 2) The mutation caused a general conformation change that only affected some of the substrates, because the affected substrates have different binding sites/translocation pathways. The effect of mutation S334K was also substrate dependent. For DPDPE and BSP, the decreased transport activity (50% - 60% of wild-type Oatp1a4) was consistent with the extent of decreased surface expression (about 50% of wild-type Oatp1a4). For taurocholate and estradiol-17 β -glucuronide, similarly as for digoxin, the transport activity was almost abolished. Therefore, in addition to surface expression, S334K also affected the transport rate of digoxin, taurocholate, and estradiol-17 β -glucuronide, but not of DPDPE or BSP. And finally, consistent with the very low surface expression level of M361S, none of the substrates were transported by this mutant.

Our data do not address whether these amino acid residues are directly involved in substrate recognition or translocation. However, the bulkiness of the side chains, *e.g.* in the amino acid residues methionine at position 361 (M361) and phenylalanine at position 328 (F328) could be important to maintain the protein in a conformation optimal for stability and substrate transport. Its loss would then lead to altered transport functions as suggested in an earlier chimeric analysis of the glucose transporters (Hxt1 and Hxt2) in yeast (Kasahara et al., 2004). A previous study aiming to generate a theoretical structural model representative of OATP/*SLCO* superfamily members (Meier-Abt et al., 2005), provides some insights concerning the location of the individual amino acids examined in the present study. The three sites (F328, S334 and M361) were all facing the putative binding pore in the theoretical models, and may thus be expected to affect the transport pathway directly. However, kinetic analysis for the mutants with measurable transport is needed to determine whether these amino acids affect the apparent affinity (K_m) constant and/or the maximal transport rate (V_{max}).

III. OVERALL CONCLUSION

Because OATP/Oatps are multi-specific transporters, identifying the domains that are important for Oatp1a4 mediated transport using different substrates will provide valuable information to further understand how the transporter members in this superfamily translocate these structurally unrelated compounds across the plasma membrane. Using chimeric and mutant Oatp1a4 protein, we evaluated the Oatp1a4-

mediated transport of five different substrates. Our study indicates that (1) the C-terminal half of Oatp1a4 is important for the transport of four substrates including digoxin, taurocholate, estradiol-17 β -glucuronide, and BSP; (2) EL4 and TM8 may be important in constructing the binding/ translocation interface of the transporter with substrates, and (3) Oatp1a4 might have different binding/translocation pathways for different substrates. These data, combined with previous findings by other groups provide further understanding of OATP/Oatp mediated transport of a broad spectrum of substrates. However, to characterize and explain the underlying molecular mechanisms, additional experiments including crystallization of the substrate-transporter complexes, in combination with functional characterization of point mutations of critical residues, are required.

CHAPTER 5

CHARATERIZATION OF RAT RENAL ORGANIC ANION TRANSPORTERS IN TRANSPORTING PERFLUORINATED CARBOXYLATES WITH DIFFERENT CHAIN LENGTHS

SPECIFIC AIM AND HYPOTHESIS

Chapter 5 will address specific aim 3 and will evaluate the hypothesis that **different rat renal organic anion transporters of the Oat and Oatp families selectively transport PFCAs depending on the chain lengths.** The purpose of this study is **to determine the size selectivity of Oats and Oatp1a1 in PFCA transport.** Furthermore, with the previous *in vivo* observation of PFCA disposition patterns and the gender specific expression of Oats and Oatps in kidney, the findings of this study also highlight the important role of transporters in renal excretion of PFCAs. The roles of Oats and Oatps in the gender-dependent renal elimination pattern of PFCAs with different chain length are thoroughly discussed in this chapter. Additional discussion in Chapter 6 will address the size selectivity of the “binding pocket” in OATs/Oats and OATP/Oatps.

I. INTRODUCTION

Perfluorinated carboxylates (PFCAs) are fatty acid analogues with fully fluorinated carbon back-bones. A representative structure of perfluorooctanoate (C8) is shown in Figure 5-1. The chain lengths for most commercially available PFCAs

range from 2 to 18 carbons (C2 – C18). PFCAs are generally stable to metabolic and environmental degradation and are found at low concentrations in samples of environmental and biological media. Although trifluoroacetate (C2) can occur naturally, C2 and most other perfluorinated carboxylates are present in the environment as a result of various commercial uses. PFCAs can also arise from degradation of other polyfluorinated chemicals (D'Eon et al., 2006; Fasano et al., 2006; Wallington et al., 2006; D'Eon and Mabury, 2007) and are more resistant to natural degradation than hydrocarbon carboxylates, due to the presence of multiple carbon-fluorine bonds (Lemal, 2004). Both PFOA (C8) and PFDA (C10) have been shown to be potent PPAR α activators and their toxicological effects observed in rodents and the potential concerns for humans have been well summarized in several reviews (Vanden Heuvel, 1996; Kudo and Kawashima, 2003; Kennedy et al., 2004). Renal clearance of PFCAs is influenced by chain length, isomeric structure (branched vs. linear), species, and, in some cases, gender within a species (Vanden Heuvel et al., 1991; Kudo and Kawashima, 2003; Butenhoff et al., 2004; Kennedy et al., 2004; Chang et al., 2008; Chengelis et al., 2009; De Silva et al., 2009).

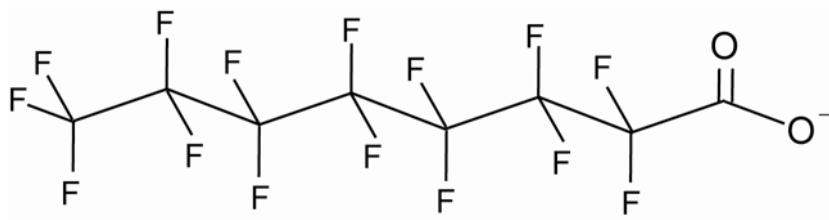


Figure 5-1. Structure of PFOA (C8)

As an example of the differences that can occur in elimination kinetics, in humans, the geometric mean half-life of perfluorooctanoate (C8) serum elimination in a group of retired production workers was 3.5 years (95% CI, 3.0–4.1) with no obvious gender differences (Olsen et al., 2007). This compares to cynomolgus monkeys, for which the serum C8 elimination half-life is estimated to be several weeks (Butenhoff et al., 2004). However, in male rats, the serum elimination half-life of C8 is several days, as compared to hours for female rats (Kudo and Kawashima, 2003; Kennedy et al., 2004). In contrast, the estimated elimination half-life of perfluorobutyrate (C4) is approximately 3 to 4 days for humans and 1.5 days for cynomolgus monkeys (Chang et al., 2008), again with no obvious gender differences in either species. Unpublished preliminary data have shown that in male and female rats exposed to C4, serum elimination half-lives are approximately 6 to 9 and 2 to 4 hrs, respectively.

Because PFCAs are not known to undergo metabolic conversion (Lau et al., 2007), differences in elimination kinetics are hypothesized to be due to differences in renal elimination as a result of differences in renal transport processes (Kudo et al., 2001; Katakura et al., 2007; Nakagawa et al., 2008; Tan et al., 2008; Nakagawa et al., 2009). Since the rat is one of the most commonly used species in toxicological studies, it is important to identify the transport systems involved in the renal

elimination of PFCAs to further understand potential toxicological effects and extrapolate the findings to humans.

The renal organic anion transport system is a very important system in facilitating the daily elimination of toxic compounds and reabsorption of useful anionic metabolites. There are several major transporter families that are involved in the transport of organic anions in kidneys: the organic anion transporter (Oat) family, the organic anion transporting polypeptide (Oatp) superfamily, the multidrug resistance protein (Mdr), and the multidrug resistance-associated protein (Mrp) superfamilies (Sekine et al., 2006). In the rat proximal tubule, these transporters are expressed in a polarized manner with specific transporters being differentially localized in the basolateral membrane and apical membrane. Transporters localized at the basolateral membrane include Oat1, Oat3 (Tojo et al., 1999; Kojima et al., 2002), and Oatp4c1 (Mikkaichi et al., 2004). Transporters localized at the brush-border membrane include Oat2 (Kojima et al., 2002), Urat1 (Rizwan and Burckhardt, 2007), Oat5 (Anzai et al., 2005), Oatp1a1 (Bergwerk et al., 1996), Mdr1a/b (Sekine et al., 2006), Mrp2 (Schaub et al., 1997), and Mrp4 (van Aubel et al., 2002). Gender-dependent expression patterns occur with several of these transporters, including Oat1, Oat2, Oat3 (Buist et al., 2002; Cerrutti et al., 2002; Ljubojevic et al., 2004; Ljubojevic et al., 2007), Oatp1a1 (Lu et al., 1996; Kato et al., 2002), and Mdr1b (Lu and Klaassen, 2008). Among these transporters, Oat1, Oat3, and Oatp1a1 have been

shown to transport C8 (Katakura et al., 2007; Nakagawa et al., 2008; Yang et al., 2009).

Given the *in vivo* evidence that there are gender dependent renal elimination patterns for PFCAs with chain lengths from 7 to 10 (Kudo et al., 2001), we hypothesized that PFCAs with different chain lengths are substrates of different organic anion transporters, and that the location and gender specific expression of those transporters contribute to the renal elimination pattern. In this study, we investigated the roles of the five rat renal organic anion transporters (Oat1, Oat2, Oat3, Urat1, and Oatp1a1) in transporting PFCAs of various chain lengths (C2 – C18). Initial experiments studied the potential of C2 – C18 PFCAs to inhibit known substrates of the transporters. Based on those results, PFCA uptake and transport kinetic studies were performed, focusing on four PFCAs (C7 – C10).

II. RESULTS

2.1 Oat1- and Oat3-mediated transport of PFCAs.

In HEK293 cells transiently transfected with rat Oat1, transport of the model substrate [³H]-*p*-aminohippurate (PAH) was strongly inhibited by 10μM of C7, followed by C6 and C8 (Figure 5-2A), whereas other homologs did not show significant inhibition at this concentration. In HEK293 cells expressing Oat3, transport of the model substrate [³H]-estrone-3-sulfate (E-3-S) was strongly inhibited by 10 μM C8

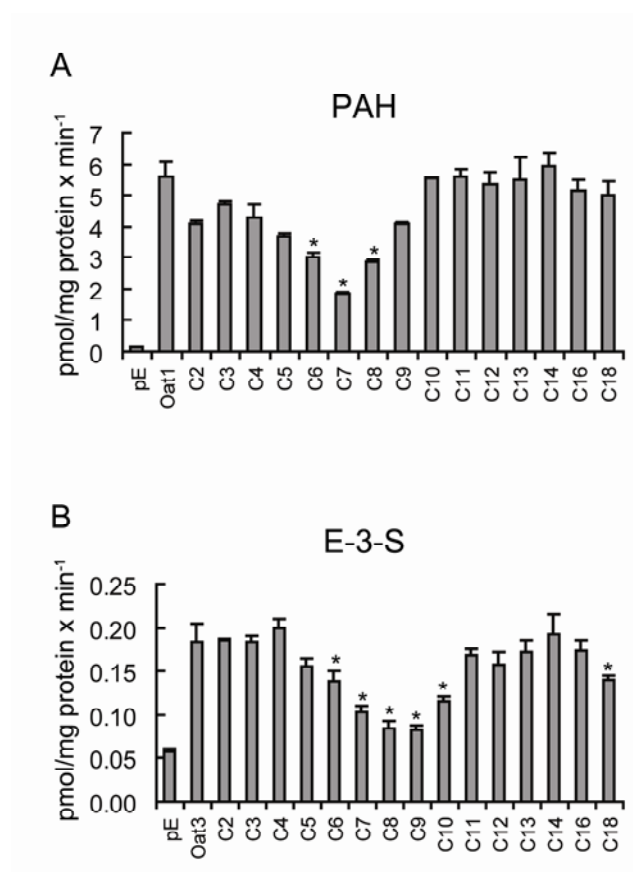


Figure 5-2. Inhibitory effect of PFCAs on Oat1 and Oat3 mediated transport.

Oat1-mediated 88nM [³H]-*p*-aminohippurate (PAH) (A) or Oat3-mediated 7nM [³H]-estrone-3-sulfate (E-3-S), (B) uptake was quantified at 37 °C for 1 min in the absence or presence of 10μM PFCAs with chain lengths from two (C2) to eighteen (C18) carbon atoms in Oat expressing or empty vector pExpress-1 (pE) transfected HEK293 cells. The results were corrected for total protein concentration in each well. Each bar is the mean ± standard deviation of triplicates. *, *p*<0.05 compared to Oat-mediated uptake in the absence of PFCAs.

and C9, followed by C7 and C10 (Figure 5-2B), whereas other homologs did not show significant inhibition at this concentration. In both cases, uptake of model substrates into empty vector transfected HEK293 cells was not affected by the presence of PFCAs (data not shown).

Based on the results of inhibition studies, Oat1- and Oat3-mediated uptake of C7, C8, C9, and C10 into Oat1- and Oat3-expressing HEK293 cells was quantified using LC-MS/MS. These results were obtained at a single time point (1 min) and at a single concentration (10 μ M). As shown in Figure 5-3A, uptake by Oat1 was highest for C7 and C8, followed by C9, whereas uptake of C10 was the lowest. Uptake into Oat3 expressing cells was highest for C9, followed by C8, C7, and C10 (Figure 5-3B).

In order to further understand how well these compounds are transported by Oat1 and Oat3, as well as whether these two transporters are important in the renal elimination of C8 and C9, Oat1 mediated C7 and C8 as well as Oat3 mediated C8 and C9 transport was further characterized. First, time-dependent uptake at low and high substrate concentrations was determined. At a low concentration (10 μ M), uptake mediated by Oat1 appeared linear up to 1.5 min for both C7 and C8 (Figure 5-4A and B), whereas uptake of C8 and C9 mediated by Oat3 appeared linear up to 2 min (Figure 5-4C and D). At a high concentration (300 μ M), uptake of C7 and C8 by Oat1 as well as C8 and C9 by Oat3 appeared linear up to 1 min (data not shown). Therefore, for kinetic analysis, all concentration-dependent uptakes were quantified at the 1 min time

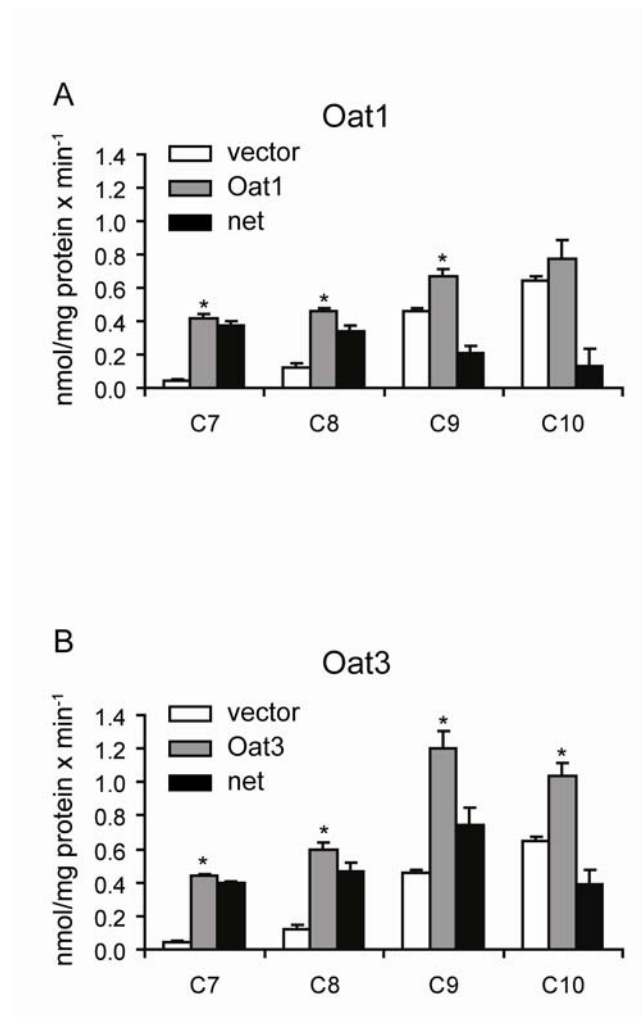


Figure 5-3. Oat1 (A) and Oat3 (B) mediated uptake of C7, C8, C9 and C10.

Uptake of 10 μ M C7 to C10 was quantified at 37 °C for 1 min with empty vector transfected (white) or Oat expressing (grey) HEK293 cells. Black bars indicate net uptake after subtracting the values of empty vector transfected cells from Oat-expressing cells. Uptake values were corrected for total protein concentration in each well. Each bar represents the mean \pm standard deviation of triplicates. *, $p < 0.05$ compared to vector control.

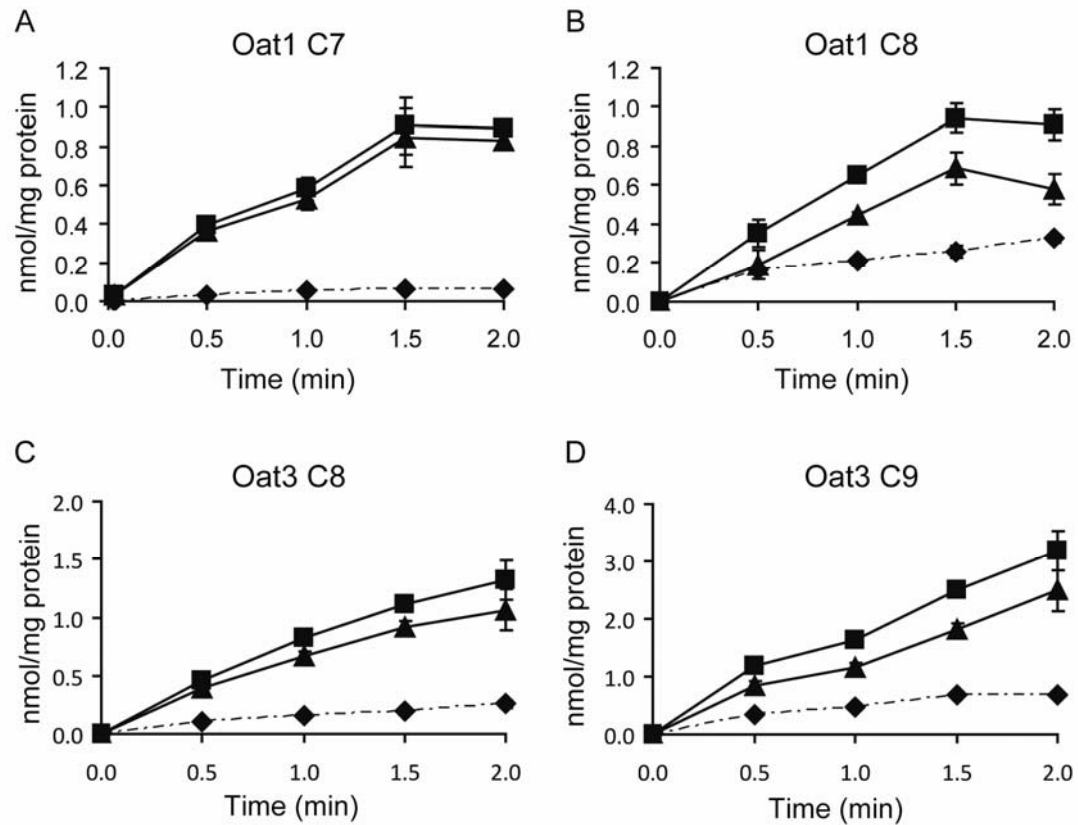


Figure 5-4. Oat1 (A, B) and Oat3 (C, D) mediated time dependent transport of PFCAs.

Uptake of 10 μ M PFCAs was quantified at 37 °C at the indicated time points with empty vector transfected (◆) or Oat expressing (■) HEK293 cells. Net uptake values (▲) were obtained by subtracting the uptake values of empty vector transfected cells from Oat expressing cells. Each point represents the mean \pm standard deviation of triplicates.

point. Concentration-dependent transport of PFCAs by Oat1 and Oat3 is shown in Figure 5-5. Kinetic parameters were calculated based on the Michaelis–Menten equation, and the K_m and V_{max} values are summarized in Table 5-1. The apparent K_m values for Oat1-mediated transport of C7 (50.5 μM) and C8 (43.2 μM) were very similar. The K_m value for Oat3-mediated C8 uptake (65.7 μM) was lower than for C9 (175 μM), but the V_{max} value of C9 uptake (8.7 $\text{nmol/mg} \times \text{min}^{-1}$) was higher than that of C8 (3.8 $\text{nmol/mg} \times \text{min}^{-1}$). Therefore, although there were some differences in the affinities, both transporters had similar efficiencies (V_{max}/K_m) for both substrates that were quantified.

2.2 Oatp1a1-mediated transport of PFCAs.

In the CHO cell line stably expressing rat Oatp1a1, transport of the model substrate [^3H]-estradiol-17 β -glucuronide (E-17 β -G) was inhibited by 10 μM of C9, C10, and C11 by 20 to 40% (Figure 5-6A), whereas uptake of model substrate into CHO-wild-type cells was not affected by the presence of PFCAs (data not shown). Next, uptake of 10 μM C7, C8, C9, and C10 into CHO-Oatp1a1 expressing cells was compared. Uptake of C9 and C10 was highest followed by C8, with minimal uptake of C7 (Figure 5-6B). Therefore, saturation kinetics for Oatp1a1-mediated transport were determined for C8, C9, and C10. Figure 5-7 A to C shows that transport of C8, C9, and C10 mediated by Oatp1a1 appeared linear up to 2 min at the 10 μM concentration, whereas at the high concentration (300 μM), uptake was only linear up to 1 min (data

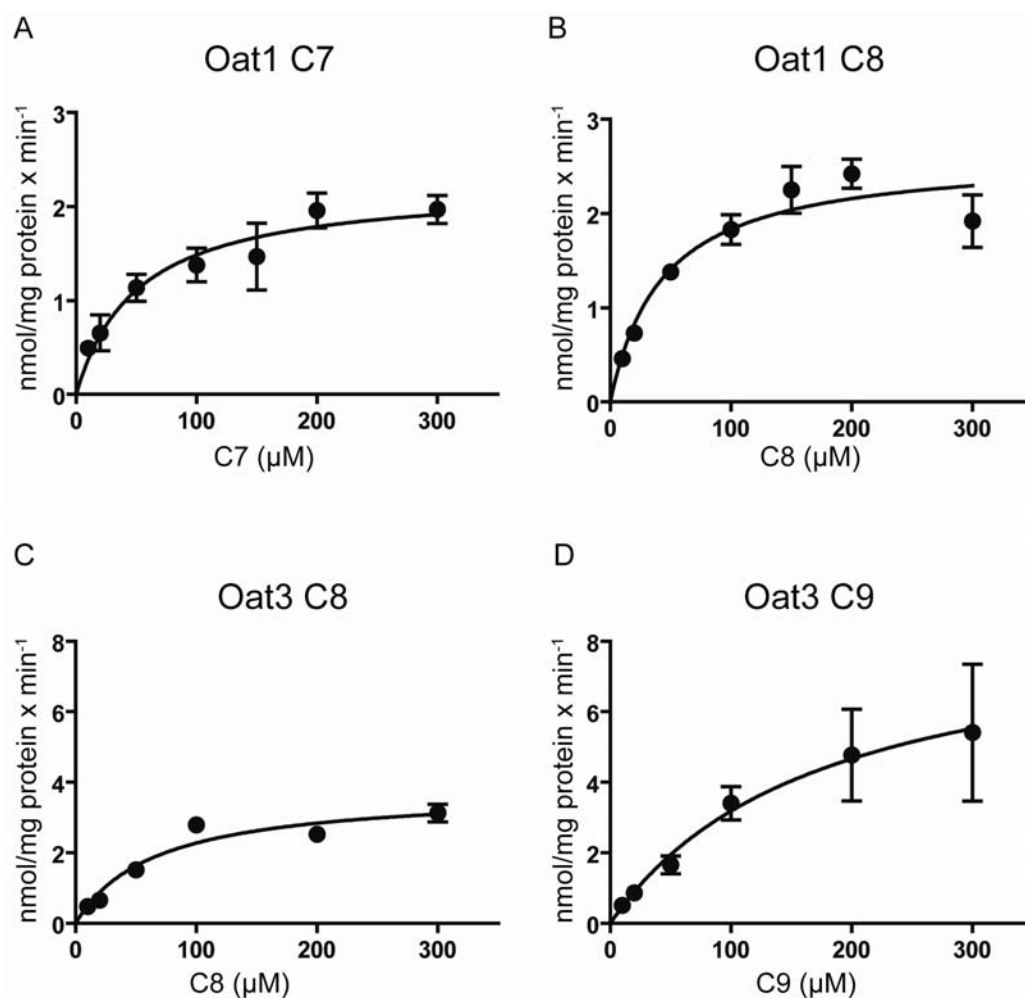


Figure 5-5. Kinetics of Oat1 (A, B) and Oat3 (C, D) mediated transport of PFCAs.

Uptake of increasing concentrations of PFCAs was quantified under initial linear rate conditions at 37°C with Oat-expressing and empty vector transfected HEK293 cells.

After subtracting the values obtained with the empty vector transfected HEK293, and corrected for total protein concentration, net Oat-mediated uptake was fitted to the Michaelis-Menten equation to obtain K_m and V_{max} values. Each point represents the mean \pm standard error from a pool of two independent experiments with triplicates.

Table 5-1: Kinetic parameters of Oat1 and Oat3 mediated PFCA transport

Transporter	PFCA	K_m	V_{max}	V_{max}/K_m
		(μM)	(nmol/mg protein x min ⁻¹)	(mL/mg protein x min ⁻¹)
Oat1	C7	50.5 \pm 13.9	2.2 \pm 0.2	0.04 \pm 0.01
	C8	43.2 \pm 15.5	2.6 \pm 0.3	0.06 \pm 0.02
Oat3	C8	65.7 \pm 12.1	3.8 \pm 0.5	0.06 \pm 0.01
	C9	174.5 \pm 32.4	8.7 \pm 0.8	0.05 \pm 0.01

Figure 5-6. Inhibition of Oatp1a1 mediated transport and uptake of selected PFCAs by Oatp1a1.

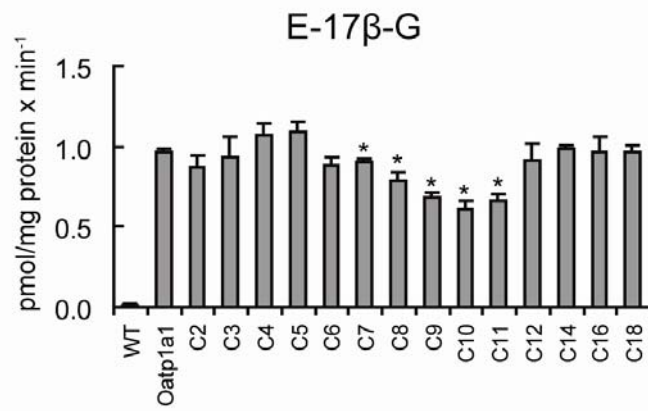
(A) Uptake of 10nM estradiol-17 β -glucuronide (E-17 β -G) was quantified at 37 °C for 1 min with Oatp1a1 expressing or wild-type CHO cells (WT) in the absence or presence of 10 μ M PFCAs with chain lengths from two (C2) to eighteen (C18) carbon atoms.

Each bar represents the mean \pm standard deviation of triplicate samples *, $p < 0.05$

compared to Oatp1a1-mediated uptake in the absence of PFCAs. (B) Uptake of 10 μ M

C7 to C10 was quantified at 37 °C for 1 min with wild-type (open bars) or Oatp1a1 expressing (grey bars) CHO cells. Black bars indicate net uptake after subtracting the uptake values of wild-type from Oatp1a1 expressing cells. Each bar represents the mean \pm standard deviation of triplicates. *, $p < 0.05$ compared to vector control.

A



B

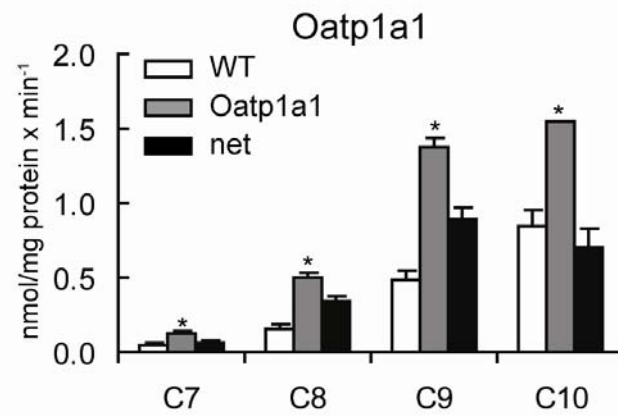
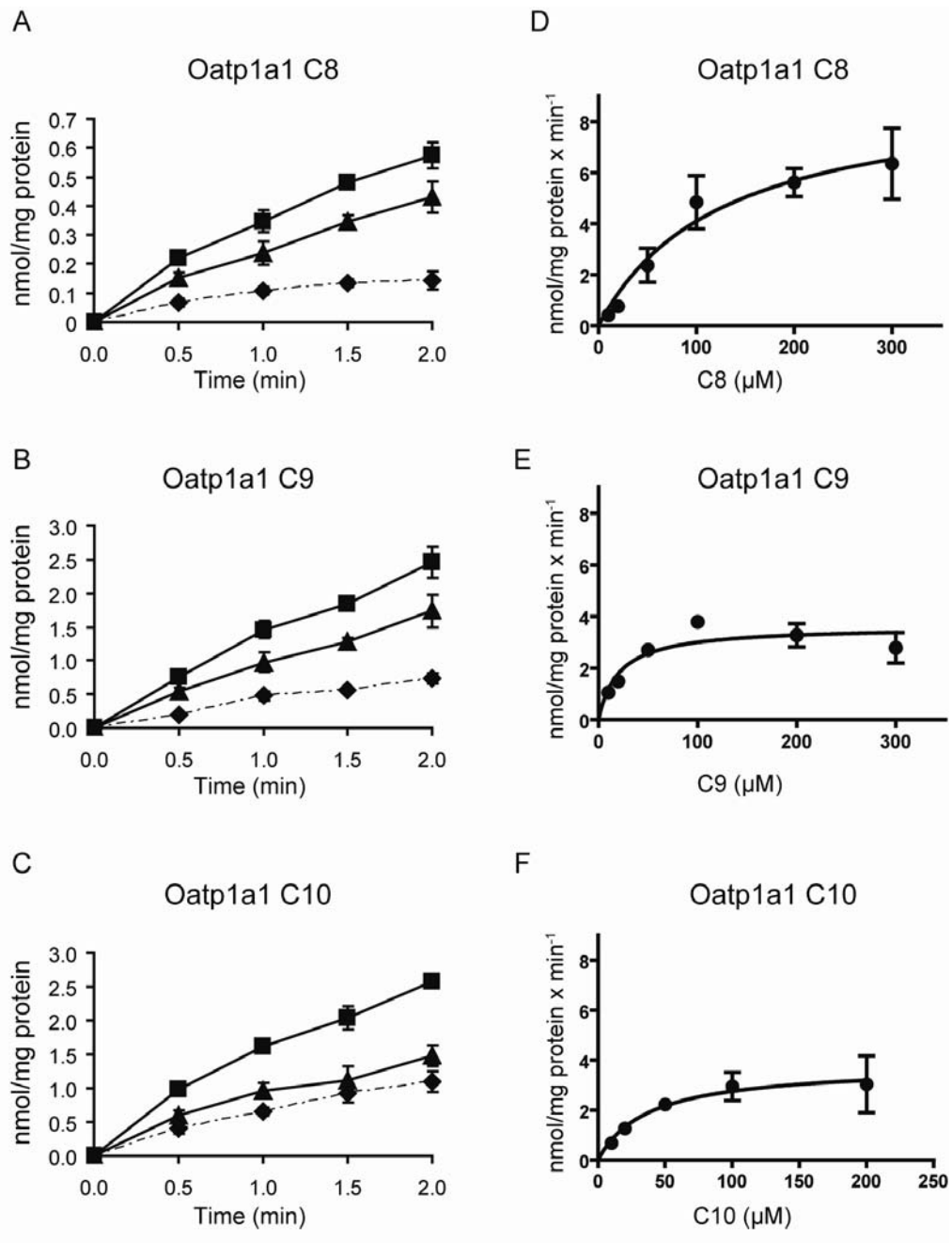


Figure 5-7. Oatp1a1 mediated time and concentration dependent transport of PFCAs.

Uptake of 10 μ M C8 (A), C9 (B) and C10 (C) was quantified at 37 °C at the indicated time points with wild-type (◆) or Oatp1a1 expressing (■) CHO cells. Net uptake values (▲) were obtained by subtracting the uptake values of wild-type from Oatp1a1 expressing cells. Each point represents the mean \pm standard deviation of triplicates. Uptake of increasing concentrations of C8 (D), C9 (E) and C10 (F) was quantified under initial linear rate conditions at 37°C. Net Oatp1a1-mediated uptake was fit to the Michaelis-Menten equation to obtain K_m and V_{max} values. Each point represents the mean \pm standard error from a pool of two independent experiments with triplicates.



not shown). As a result, all concentration-dependent uptakes for Oatp1a1 were quantified at 1 min. Figure 5-7 D to F show saturation kinetics for the three substrates, and the calculated parameters are summarized in Table 5-2. The K_m value of 126.4 μM for Oatp1a1-mediated C8 transport was about six-fold higher than the value for C9 (20.5 μM) and four fold higher than the value for C10 (28.5 μM). Taking the V_{\max} values into account, the overall transport efficiency for C8 uptake was about one half that for C9 and C10 transport.

2.3 Oat2- and Urat1-mediated PFCA transport.

In Flp-In CHO cells stably transfected with Oat2, transport of the model substrate [^3H]-PAH (Figure 5-8A) was inhibited 40% to 60% by most of the PFCAs at 10 μM . However, the chain-length-dependent inhibition pattern observed for Oat1-, Oat3-, and Oatp1a1-mediated transport was not seen for Oat2. Very similar results were obtained when the concentrations of the PFCAs were increased to 100 μM (data not shown). In HEK293 cells transiently transfected with rat Urat1, [^{14}C]-uric acid transport was not inhibited strongly by any of the tested PFCAs at 10 μM (Figure 5-8B). After increasing the inhibitor concentration to 100 μM , C8 showed about 30% inhibition, whereas there was no difference for C7, C9, and C10 (data not shown). Direct uptake of 100 μM C7, C8, C9, and C10 was determined in Oat2-expressing CHO cells, and compared to wild-type CHO cells. No significant net Oat2-mediated uptake could be seen (Figure 5-9A). Similarly, direct uptake of 100 μM C7, C9, and C10 into Urat1- expressing HEK293 cells (Figure 5-9B) was very similar to empty vector transfected HEK293 cells,

Table 5-2: Kinetic parameters of Oatp1a1 mediated PFCA transport

Transporter	PFCA	K_m	V_{max}	V_{max}/K_m
		(μM)	(nmol/mg protein x min ⁻¹)	(mL/mg protein x min ⁻¹)
Oatp1a1	C8	126.4 \pm 23.9	9.3 \pm 1.4	0.07 \pm 0.02
	C9	20.5 \pm 6.8	3.6 \pm 0.5	0.18 \pm 0.06
	C10	28.5 \pm 5.6	3.8 \pm 0.3	0.13 \pm 0.03

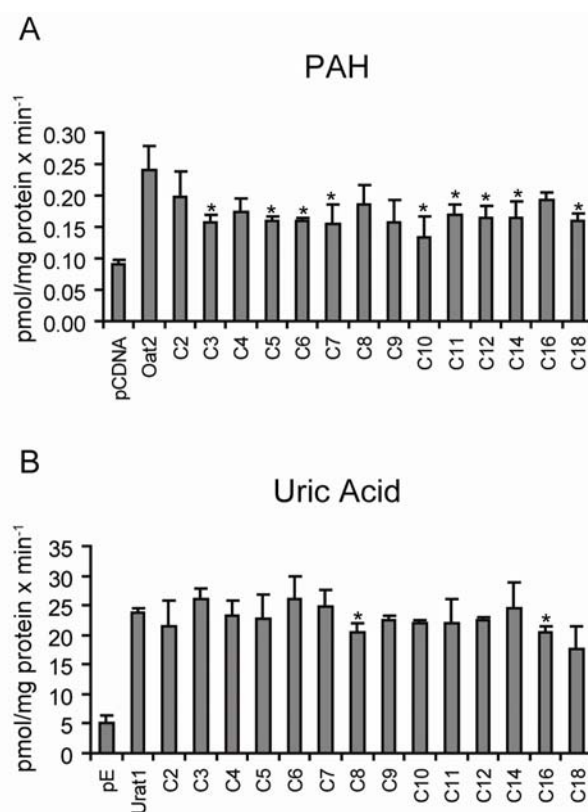


Figure 5-8. Effect of PFCAs on Oat2 and Urat1 mediated transport.

Uptake of Oat2-mediated 88nM [³H] – PAH (A) or Urat1-mediated 10μM [¹⁴C]- uric acid (B) uptake was quantified at 37 °C for 1 min in the absence or presence of 10μM PFCAs with chain lengths from two (C2) to eighteen (C18) carbon atoms. Each bar represents the mean ± standard deviation of triplicates. *, *p*<0.05, compared to Oat2 or Urat1-mediated uptake in the absence of PFCAs.

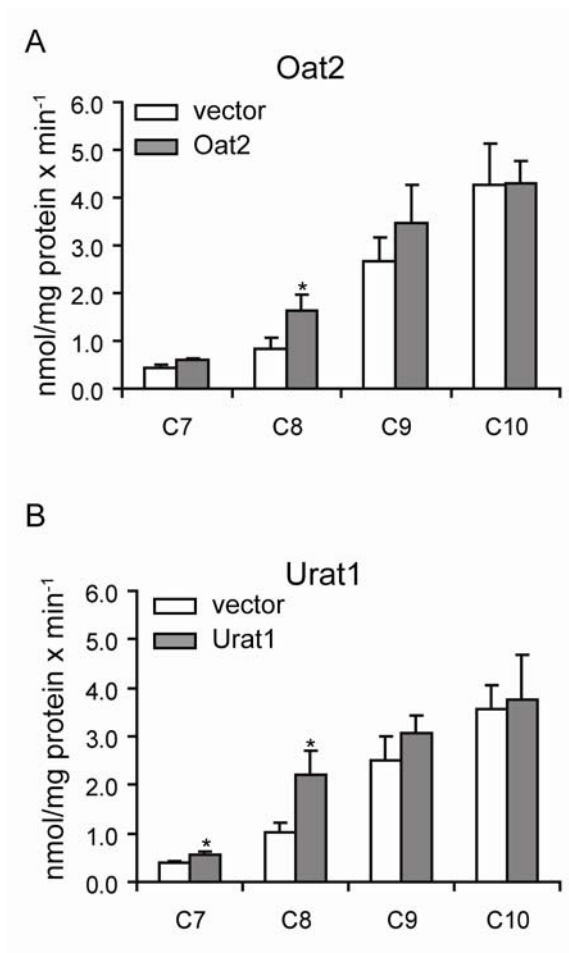


Figure 5-9. Oat2 (A) and Urat1 (B) mediated uptake of C7, C8, C9 and C10.

Uptake of 100 μ M C7 to C10 was measured at 37 °C for 1 min with empty vector transfected (white bars) or transporter-expressing (grey bars) HEK293 cells.

Concentrations of PFCAs were corrected for total protein concentration in each well.

Each bar represents the mean \pm standard deviation of triplicates. *, $p < 0.05$, compared to vector control.

whereas uptake of C8 was approximately a third higher for Urat1-expressing cells, consistent with the inhibition in [^{14}C]-uric acid observed at 100 μM C8.

III. DISCUSSION:

A gender-dependent elimination pattern of PFCAs with different chain lengths has been reported in rats *in vivo* (Kudo et al., 2001). In the present study, we have demonstrated that, depending on the chain length, PFCAs from C7 to C10 are substrates of three renal organic anion transporters: Oat1, Oat3, and Oatp1a1. C8 is a substrate of Oat2 and Urat1 (Figure 5-10). We have also demonstrated that C7 and C8 have the highest affinities for Oat1 and Oat3, whereas C9 and C10 have the highest affinities for Oatp1a1.

Both Oat1 and Oat3 have been reported to be expressed at the basolateral membrane (Kojima et al., 2002); (Tojo et al., 1999) and function as organic anion/dicarboxylate exchangers (Sekine et al., 1997; Sweet et al., 1997; Wolff et al., 1997; Bakhiya et al., 2003; Sweet et al., 2003). Based on the estimation that greater than 90% of PFOA is bound to serum albumin (Han et al., 2003), Oat1 and Oat3 mediated excretion may play an important role in renal elimination of PFCAs (Figure 5-10). This is further supported by a study that showed that pre-treatment with probenecid, which inhibits Oat1 and Oat3, reduces renal clearance of PFOA (Kudo et al., 2002). Katakura *et al.* (Katakura et al., 2007) showed that Oat3-transfected *Xenopus laevis* oocytes

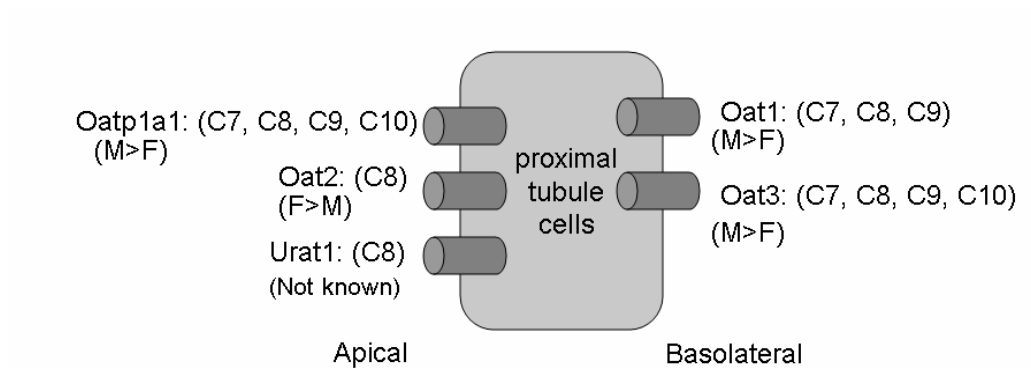


Figure 5-10. Diagram of PFCA transport in kidney. M>F: the expression level of the indicated transporter is higher in male rats; F>M: the expression level of the indicated transporter is higher in female rats. C7, C8, C9 and C10 in parentheses: substrates of the indicated transporter.

transport C8. The apparent K_m values of Oat1 and Oat3-mediated C8 transport in our study are very close to previously published apparent K_m values (51 μ M for Oat1 and 80 μ M for Oat3, respectively) (Nakagawa et al., 2008). We also found that Oat1 has very similar transport efficiencies for C7 and C8. Oat3 has similar overall transport efficiency for C8 and C9. Moreover, our results indicate that compared to Oat1, Oat3 is better at transporting PFCAs with longer carbon chains (C9 and C10). Although gender-dependent expression has been reported for both Oat1 and Oat3 (Cerrutti et al., 2002; Ljubojevic et al., 2004), the rats used in the PFOA renal elimination study done by Kudo *et al.* did not show marked differences at the mRNA level for either Oat1 or Oat3 (Kudo et al., 2002). This could be due to the rats used in study by Kudo *et al.* was much younger than the rats used in the study by Cerrutti *et al.* and Ljubojevic *et al.*. Thus, available data suggest that Oat1 and Oat3 may play an important role in the secretion of PFCAs from plasma into proximal tubule cells, and that additional transporters must be involved in the observed gender-dependent renal excretion pattern.

Oatp1a1 is an important transporter expressed on the apical membrane of proximal tubule cells with a male dominant expression pattern (Bergwerk et al., 1996; Kato et al., 2002; Kudo et al., 2002). It mediates reabsorption of organic anions in exchange for intracellular reduced glutathione (Li et al., 1998). Katakura *et al.* demonstrated that Oatp1a1-expressing *Xenopus laevis* oocytes transported C8 (Katakura et al., 2007) and Yang *et al.* (Yang et al., 2009) extended these studies and determined that the apparent K_m value for Oatp1a1-mediated C8 transport is 162 μ M.

Furthermore, they calculated apparent inhibition constants for estrone-3-sulfate uptake by C6, C7, C8, C9 and C10. Based on these inhibition constants, and assuming that in addition to C8, other PFCAs are also substrates of Oatp1a1, they predicted that PFCAs with longer chain lengths would have higher affinities (apparent K_i values for C6: 1858 μM ; C7: 399 μM ; C8: 84 μM ; C9: 45 μM ; C10: 27 μM). In this present study, we determined directly the apparent K_m values for C8 (126 μM), C9 (20 μM) and C10 (28 μM). The apparent K_m value for C8 of 126 μM determined in this study is comparable to the apparent K_m value of 162 μM described by Yang *et al.* (Yang et al., 2009). Furthermore, the apparent K_m value of 28 μM for C10 determined in this study is similar to the apparent K_i value of 28 μM (Yang et al., 2009). However, the predicted affinities by Yang *et al.* for C8 (84 μM) is much lower than the values quantified by Yang *et al.* (162 μM) (Yang et al., 2009) and by us (126 μM), and the predicted affinities for C9 (45 μM) is higher than the values quantified by us (21 μM) .

The significant differences in the K_m values that we quantified in this study for Oatp1a1-mediated C8, C9 and C10 uptake suggest that Oatp1a1 transports C10 and C9 with higher efficiency (V_{max}/K_m) than C8. Because Oatp substrates are mainly anionic amphipathic molecules with high molecular weight (>450) that under normal physiological conditions are bound to albumin (Hagenbuch and Meier, 2003), it is not surprising that PFDA (MW 514) and PFNA (MW 464) are better substrates for Oatp1a1 than PFOA (MW 414). Furthermore, the strong reabsorption mediated by Oatp1a1 for PFCAs with greater chain length may also explain the fact that the longer

the carbon chain, the less PFCA is excreted into the urine (Kudo et al., 2001). Because male rats have more Oatp1a1 present at the apical membrane, it is reasonable that in male rats less C8 and C9 are excreted into the urine than in female rats as was shown previously (Kudo et al., 2001).

In this study, we also investigated the role of two other apical organic anion transporters, Oat2 and Urat1. Oat2 has a female dominant expression pattern in the rat at the apical membrane of proximal tubule cells. However, in humans, this transporter is localized to the basolateral membrane (Enomoto et al., 2002b; Kojima et al., 2002; Ljubojevic et al., 2007). Although most PFCA tested in this study inhibited Oat2-mediated PAH uptake by 40% to 60%, the chain length dependent inhibition pattern as shown for Oat1, Oat3 and Oatp1a1 mediated transport was not seen. The general inhibition of PFCAs observed could be due to the low signal to noise ratio because the amount of PAH transported into Oat2-expressing cells is only about twice as much as that of wild-type cells.

Furthermore, direct transport of C7 to C10 into Oat2-expressing CHO cells did not show any difference from wild-type CHO cells. The same result was obtained when we tested the direct transport of C7 to C10 in HEK cells transiently transfected with rat Oat2 (data not shown). Thus, we conclude that in our experimental systems, Oat2 does not transport C7, C8, C9 or C10, which for C8 is consistent with the previous finding by Nakagawa *et al.* (Nakagawa et al., 2008) that Oat2 does not transport C8 in

transiently transfected HEK cells. Similar to Oat2, Urat1 did not show any chain length dependent inhibition pattern. Although C8 transport into Urat1 expressing HEK293 cells was slightly higher than empty vector transfected cells, the signal to noise ratio was not large enough for further characterization. Among the three transporters expressed at the apical membrane, Oatp1a1 is the major player in the reabsorption of PFCAs.

There are also efflux transporters expressed at the apical membrane, including Mdr1a/1b and Mrps which may play a role in active secretion of PFCAs into urine. Mdr1b is much higher expressed in female rats at the mRNA level (Lu and Klaassen, 2008) and could therefore potentially affect secretion of PFCAs across the apical membrane in female rats. Given the complexity of transporter systems expressed in the kidney, further studies are needed to elucidate PFCA excretion from proximal tubule cells into the tubular lumen.

The roles of several human transporters in renal clearance of C8 have been studied by other groups. It has been shown that human Oat1 and Oat3 have similar C8 transport capacity compared to the rodent homologues (Nakagawa et al., 2008). The same group also showed that a human specific transporter hOAT4 also transports C8 (Nakagawa et al., 2009). The functional studies of rat transporters by us and others combined with previously published *in vivo* toxicokinetics experiments will allow us to generate additional human transporter candidates that may play important roles in

PFCAs disposition. For example, Oatp1a1 has been shown in current and previous studies to be important in reabsorption of C8, C9 and C10 in rats. It would be very interesting to look at the role of human OATP1A2, which is the orthologue of rat Oatp1a1, in transporting PFCAs with different chain lengths. Such studies will also provide valuable experimental kinetic parameters for toxicokinetic modelling of PFCAs in humans.

In conclusion, our studies reveal that PFCAs with different chain lengths are substrates of the two basolateral transporters, Oat1 and Oat3, as well as of the apical transporter, Oatp1a1, in rat proximal tubule cells. The male dominant expression level of Oatp1a1 in kidney as well as differences in transport efficiencies for PFCAs with different chain lengths suggests a role of Oatp1a1 in the gender-specific renal elimination pattern in rats. Further research is required to understand the basis of potential species differences in organic-anion transporter-mediated transport of PFCAs and how these may relate to species differences in elimination kinetics that have been observed.

CHAPTER 6

SUMMARY AND DISCUSSION OF DISSERTATION

Organic anionic transporting polypeptides are important membrane transporters that mediate sodium-independent transport of amphipathic organic compounds in mammalian cells. Contrary to most *SLC* family members that have very specific substrate selectivity, OATP/Oatps are able to recognize and transport many structurally unrelated substrates. Together with the organic anion transporters (OATs), the organic cation transporters (OCTs), the multi-drug resistance protein (MDR), and the multi-drug resistance-associated proteins (MRPs), OATP/Oatps play an essential role in absorption, disposition, excretion and homeostasis of many physiological metabolites and foreign compounds. Malfunction of OATPs, due to genetic polymorphisms can lead to decreased drug efficacy, and more importantly, increased toxicity. Abnormal expression patterns of OATPs have also been reported in several cancers. Therefore, for better human health it is important to understand how these transporters function and how they are regulated.

Prior to this dissertation work, very little was known about structure-function relationship of OATP/Oatps. It was suggested that the C-terminal half of human OATP2A1 may determine the transport affinity for prostaglandin E₂ by using chimeras

between human and mouse proteins (Pucci et al., 1999). Cysteine scanning was used to map the substrate binding site of OATP2A1 and TM10 has been shown to be involved at least partially in substrate binding (Chan et al., 1999). The same group also studied the roles of conserved transmembrane cationic amino acids in OATP2A1 mediated transport, and R560 in TM11 was identified to be important in substrate translocation (Chan et al., 2002). In 2005, theoretical 3-D structural models for the OATP superfamily members OATP1B3 and OATP2B1 were published, in which a positively charged binding pocket was predicted by quantifying the electrostatic potential on the surface (Meier-Abt et al., 2005). The overall goal of this dissertation has been to extend those previous findings. My research has focused on the structure-function relationship of OATP/Oatps. Concurrently with my research, a few more studies with similar research interests have been published. The critical role of transmembrane domain (TM) 10 for OATP1B3-mediated transport was characterized. (Gui and Hagenbuch, 2008). Miyagawa *et al.* later demonstrated that TM8 and TM9 in OATP1B1 affects transport kinetics of estrone-3-sulfate and estradiol-17 β -glucuronide (Miyagawa et al., 2009). The importance of TM10 in OATP1B1 protein structure and transport function was further studied by Gui and Hagenbuch (2008). Although the roles of several transmembrane domains and individual amino acids for the transport function of OATP1B subfamily members have been studied in detail, very little is known about the binding/translocation sites for different substrates.

As mentioned in the introduction, I will defend three specific aims in this dissertation. In the **first specific aim**, I evaluated the hypothesis that conserved

positively charged amino acids play important roles in OATP1B1 transport function. OATP1B1 and 1B3 are closely related transport proteins that mediate translocation of a wide range of compounds across the sinusoidal membrane of hepatocytes. Using comparative modeling, based on crystal structures of major facilitator superfamily members, a putative structural model of OATP1B3 with a “Positive Binding Pocket” has been predicted (Meier-Abt *et al.*, 2005). Based on this model, we tested the hypothesis that positive amino acids, conserved in the OATP1B family and predicted to be facing this binding pocket, are important for OATP1B1 function. We performed site-directed mutagenesis and expressed the resulting constructs in HEK293 cells for functional analysis. Surface expression of wild-type and mutated transporters were quantified using surface biotinylation, followed by Western blot. Alanine substitutions of R57 (R57A) and K361 (K361A), which are located at the extracellular entrance of the putative binding pocket, greatly reduced transport activity for all three model substrates (estradiol-17 β -glucuronide, estrone-3-sulfate and bromosulfophthalein) of OATP1B1. R57 positive charge substitution (R57K) rescued transport function, but K361 positive charge substitution (K361R) did not. Substitution of the transmembrane domain R580 with alanine resulted in less than 30% surface expression compared to wild-type OATP1B1. At the intracellular side of OATP1B1, K90A moderately reduced transport activity of all three substrates and K90R successfully rescued the function. Mutations of H92 and R93 affected BSP transport more than estradiol-17 β -glucuronide or estrone-3-sulfate transport. In conclusion, conserved positively charged amino acid residues facing the putative

binding pocket of OATP1B1 are important in transport function and/or surface expression of the transporter.

Cationic amino acids, such as arginine, are not often seen within transmembrane domains. R580 as a predicted transmembrane arginine is highly conserved in all OATP/Oatp families. Chan *et al.* demonstrated that replacing R560, the arginine at the corresponding position in TM11 of OATP2A1 with the neutral asparagine abolished transport. However, substitution with the positively charged lysine resulted in a comparable K_m but a greatly decreased V_{max} value (Chan *et al.*, 2002), indicating that this arginine is important in substrate translocation. Moreover, R454 in TM11 of rOAT3 is well conserved among OAT family members, whereas at the same position of OCTs, there is a conserved negatively charged aspartic acid. When mutating R454 to D, rOAT3 lost transport activity for most model substrates except cimetidine (Feng *et al.*, 2001). The observations in both studies are consistent with the results in specific aim 1 that the V_{max} values of R580 mutants mediated estradiol-17 β -glucuronide and estrone-3-sulfate transport were greatly decreased. More interestingly, the V_{max} values of R580 mutants mediated BSP transport did not change, suggesting similar to rOAT3 mediated cimetidine transport, BSP might use an alternative translocation pathway that does not require the transmembrane arginine. The presence of multiple binding/translocation sites could, to a certain extent, explain the broad substrate specificity of OATP/Oatps and OATs.

At the extracellular and intracellular side, positively charged amino acids can function to attract anions in some transporters. One example is the CFTR (cystic fibrosis conductance regulator) chloride channel. On both sides of the channel, are multiple positively charged amino acid clusters including arginines and lysines, to attract Cl^- (Aubin and Linsdell, 2006; Zhou et al., 2008). We observed an increased K_m value by the mutation of R57A in OATP1B1. Although we do not know whether the substrates can directly bind to R57, this conserved R57 at the extracellular side of OATP1B1 might function to form a positively charged cluster with other amino acids, and attract organic anions to the extracellular mouth of the translocation pathway.

In the **second specific aim**, I evaluated the hypothesis that quantifying transport activities of different substrates mediated by chimeras between rat Oatp1a1 and Oatp1a4 in combination with site-directed mutagenesis should allow us to identify regions and/or individual amino acids that are important for Oatp1a4-mediated substrate recognition and/or transport. Most functionally characterized organic anion transporting polypeptides (Oatps) have overlapping substrate specificities and transport a wide variety of structurally unrelated compounds. Oatp1a4 is a high-affinity digoxin transporter whereas Oatp1a1 does not transport digoxin. To identify the regions that are important for Oatp1a4-mediated transport, we expressed chimeras between Oatp1a4 and Oatp1a1 in *X. laevis* oocytes and HEK293 cells and used digoxin transport as a functional tool. Based on the results with these chimeras, we

identified a region near transmembrane domain 8 as essential for digoxin transport. We then quantified uptake of the general Oatp substrates taurocholate, estradiol-17 β -glucuronide, DPDPE, and BSP in HEK293 cells expressing the chimeric proteins. In addition to digoxin, chimera 1 with the C-terminal half of Oatp1a4 also transported taurocholate, estradiol-17 β -glucuronide and BSP but not DPDPE. Consistent with findings for digoxin, replacing TM8 and EL4 of Oatp1a4 in chimera 1 with TM8 and EL4 of Oatp1a1 abolished transport activities of all substrates tested. Using site-directed mutagenesis based on sequence alignments followed by digoxin uptake we identified three amino acids (F328, S334 and M361) that when mutated to their Oatp1a1 counterparts showed reduced function. Mutant F328V did not affect transport of BSP or digoxin but showed reduced transport of taurocholate, estradiol-17 β -glucuronide, and DPDPE. Transport rates of mutant S334K for taurocholate and estradiol-17 β -glucuronide were less than 20% of the wild-type whereas BSP and DPDPE transport rates were about 50%, which is consistent with the surface expression level of this mutant. Mutant M361S mainly had decreased surface expression. In conclusion, the C-terminal half of Oatp1a4 is essential for Oatp1a4 mediated transport in a substrate dependent way, and the amino acids in TM8 and EL4 might be important for maintaining the binding/transport pathway of Oatp1a4.

Previously, the contribution of the C-terminal half to the transport function of three OATP transporters, OATP1B1 (Gui and Hagenbuch, 2009; Miyagawa et al., 2009), OATP1B3 (Gui and Hagenbuch, 2008) and OATP2A1 (Pucci et al., 1999) has

been studied. For the OATP1B subfamily, in general, the finding was that the more C-terminal transmembrane domains were retained in the chimera, the better were the specific transport activities (Gui and Hagenbuch, 2008; Gui and Hagenbuch, 2009; Miyagawa et al., 2009). For OATP2A1, the uptake and inhibition profiles of the chimeric protein with the C-terminal half of the human transporter and the N-terminal half of the mouse transporter were identical to human OATP2A1. Similarly, in our study, chimera 1, which contains the C-terminal half of Oatp1a4, was able to transport most of the Oatp1a4 substrates tested, except for DPDPE. However, chimera 2, which contains the C-terminal half of Oatp1a1 was only able to transport BSP but none of the other Oatp1a1 substrates tested. This suggests that Oatp1a1 requires some additional N-terminal portion to transport digoxin, taurocholate, and estradiol-17 β -glucuronide, whereas Oatp1a4 does not. It has been shown in the *X. laevis* oocyte system that rifampicin strongly inhibits taurocholate and estradiol-17 β -glucuronide transport mediated by Oatp1a4, but does not inhibit Oatp1a1 (Fattinger et al., 2000). This suggests that in Oatp1a1, different amino acid residues comprise the binding sites, or translocation pathways of some of the common Oatp substrates. This could explain why rifampicin only inhibited Oatp1a4 but not Oatp1a1. Thus, in order to map the domain of Oatp1a1, which is important for most of the common substrates, construction of more chimeric proteins containing additional N-terminal transmembrane domains are needed in future studies.

Chimeric proteins are powerful tools to study transport mechanism of different transporters with high amino acid sequence identity. Using a substrate specific for one transporter as a functional tool, combined with sequence comparisons to identify unique amino acid sequences, often leads to the discovery of key residues that are involved in the transport of the substrate. Usually, the two transporter pairs tested, such as OATP1B1 and OATP1B3, Oatp1a1 and Oatp1a4, or human and rodent NTCP/Ntcp, also have an overlap in their substrate spectra. Due to the high sequence identity, it is easy to hypothesize that those common substrates are probably handled through a very similar mechanism. Thus these residues that are important for the transport mechanism should be conserved and the mirror image or reciprocal chimeras should have similar effects on transport activity for common substrates. In my research I found exactly this for BSP and DPDPE transport mediated by chimera 1 and chimera 2. However, for other common substrates, such as taurocholate and estradiol-17 β -glucuronide, chimera 1 and chimera 2 behaved very differently. A conformational change of the transporter caused by the construction of chimeras might be one explanation for the different behaviors of the reciprocal constructs. However, taking into account that some modulators, such as the inhibitor rifampicin also have different effects on intact Oatp1a1 and Oatp1a4, it is possible that even for common substrates these two transporters may use different combinations of amino acid residues to form the transport pathway.

In the **third specific aim**, I evaluated the hypothesis that different rat renal organic anion transporters in the Oat and Oatp families selectively transport PFCAs of different chain lengths. PFCAs are generally stable to metabolic and environmental degradation and are found at low concentrations in environmental and biological samples. Renal clearance of PFCAs depends on chain length, species, and, in some cases, gender within species. Whereas perfluoroheptanoate (C7) is almost completely eliminated renally in both male and female rats, renal clearance of perfluorooctanoate (C8) and perfluorononanoate (C9) is much higher in female rats. Perfluorodecanoate (C10) mainly accumulates in the liver for both genders. Therefore, we tested whether PFCAs with different chain lengths are substrates of rat renal transporters with gender specific expression patterns. Inhibition of uptake of model substrates was quantified for the basolateral Oat1 and Oat3, and the apical Oat2, Oatp1a1, and Urat1 with 10 μ M PFCAs with chain lengths from 2-18 (C2 – C18) carbons. Perfluorohexanoate (C6), C7, and C8 inhibited Oat1-mediated *p*-aminohippurate transport, with C7 being the strongest inhibitor. C8 and C9 were the strongest inhibitors for Oat3-mediated estrone-3-sulfate transport, whereas Oatp1a1-mediated estradiol-17 β -glucuronide uptake was inhibited by C9, C10, and perfluoroundecanoate (C11), with C10 giving the strongest inhibition. No strong inhibitors were found for Oat2 or Urat1. Kinetic analysis was performed for the strongest inhibitors. Oat1 transported C7 and C8 with K_m values of 51 μ M and 43 μ M, respectively. Oat3 transported C8 and C9 with K_m values of 66 μ M and 175 μ M, respectively. Oatp1a1-mediated transport yielded K_m values of 126 μ M (C8), 20 μ M (C9), and 28 μ M (C10). These results suggest that

Oat1 and Oat3 are involved in the renal secretion of C7 to C9, whereas Oatp1a1 may contribute to the reabsorption of C8 through C10, with highest affinities for C9 and C10.

When using molecular weight as a standard, C10 (MW 514) and C9 (MW 464) are better substrates for Oatp1a1 than C8 (MW 414). However, the chain lengths of the linear form of PFCAs can also be used as a “ruler” to measure the actual size of the binding pocket. It is very interesting that the calculated chain length of C9 (9.6 Å) and C10 (11.6 Å) are also comparable to the calculated length of some of the Oatp1a1 substrates, such as estradiol-17 β -glucuronide (~10 Å) whereas the chain length of C8 (8.8 Å), which is a less optimal substrate, is smaller. In contrast, Oat1 and Oat3 are able to transport smaller PFCAs compared to Oatp1a1. C7 (7.3 Å) and C8 (8.8 Å) are the best sizes for Oat1 whereas C8 (8.8 Å) is a better size for Oat3 than C9 (9.6 Å). Therefore, similar to the molecular weight selectivity, Oats and Oatps also have spatial size selectivity for their substrates.

Usually, the larger the molecular weight of a molecule, the bigger is its molecular size. In this specific aim, we demonstrated that Oatp1a1 has a binding pocket that can accommodate molecules from 9 to 12 Å, whereas Oat1 and Oat3 prefer substrates around 7 to 8 Å.

There are also situations where the molecular weight of a molecule does not necessarily correlate with the size, for example, the molecules with rigid ring structures. Both uric acid and perfluoroheptanoates (C7) are substrates of Oat1. The molecular weight of uric acid is 168 whereas the molecular weight of C7 is 364. In the PFCA compound series, pentafluoropropionic acid (C3) (MW 164) has a molecular weight close to uric acid, but C3 is not a substrate of Oat1. However, due to the presence of ring structures in uric acid, the longest distance in uric acid is around 6.2 Å (Sours and Swift, 2004) which is much closer to the predicted lengths of C7 (7.3 Å) than C3 (2.9 Å). Although direct transport was not quantified in this study, C6 (6.8 Å), which is even closer in size to uric acid, inhibited Oat1-mediated uptake by 40%, and thus may also be a substrate of Oat1. Therefore, in this case, the actual size of the molecule rather than the molecular weight would be a more accurate tool for substrate prediction.

Based on inhibition constants, Yong *et al.* (2009) predicted that PFCAs with longer chain lengths would have higher affinities for Oatp1a1 mediated transport (apparent K_i values for C6: 1857 μM ; C7: 399 μM ; C8: 84 μM ; C9: 45 μM ; C10: 27 μM) (Yang et al., 2009). In our study, by quantifying the direct transport kinetics, we demonstrated that the apparent affinities of PFCA transport mediated by Oatp1a1 do not correspond linearly to the size of the molecules. For Oatp1a1, C9 and C10 have very similar K_m values (20.5 μM for C9 and 28.5 μM for C10). This is also true for Oat1 mediated C7 and C8 (50.5 μM for C7 and 43.2 μM for C8). Therefore, it seems

that the binding affinity of substrate is not affected as long as the substrate is within the size limit of “binding pocket”. However, when the size of the compound is at the upper limit of the substrate size range for a transporter, the actual size of the molecule will greatly affect the transport affinity. For example, because Oat3 has less affinity for C9 ($K_m=175\ \mu\text{M}$) than for C8 ($K_m=66\mu\text{M}$), it may indicate that the size of C9 (9.6 Å) is approaching the upper limit of the substrate size range for Oat3. Thus, linear PFCAs with different chain lengths are great tools to study the substrate size selectivity of Oats and Oatps.

Overall, in this dissertation, I examined the transport mechanism of organic anion transporting polypeptides from three perspectives. First, I investigated the roles of conserved positively charged amino acid residues. I found that some highly conserved amino acids are critical for transport function. Second, I focused on the differences between two highly similar transporters. Again, several amino acids that are important for transport function were identified. More importantly, in both studies, different results were obtained when using different substrates for functional measurements. This is consistent with previous studies, and further supports the hypothesis that OATP/Oatps have multiple binding sites/translocation pathways for their different substrates. Finally, from the perspective of substrates, I predicted the size of the binding pockets in Oat1, Oat3 and Oatp1a1 using PFCAs with different chain lengths. This work has provided valuable information for further understanding the transport mechanism of OATP/Oatps. The experimental results, including the

mutagenesis studies and the measurement of the binding pocket size, are also very helpful in testing the accuracy of future computational models. However, to fully understand the molecular mechanism of transporter functions, there is still a long way to go. With more advanced computational, molecular biological and crystallography techniques, further work is necessary to explore the more detailed structure-function relationship of membrane transporters.

CHAPTER 7

FUTURE DIRECTIONS

The studies in specific aim 1 have shown that the positively charged amino acids R57 and K361 at the extracellular side of OATP1B1 are important in substrate binding. However, we do not know whether they can directly bind the substrates or whether other adjacent amino acids are also important in facilitating substrate binding. To further investigate these two potential substrate binding domains, a cysteine scanning mutagenesis study should be employed to probe the substrate accessibility of the amino acids close to R57 and K361. Because we know that OATP1B1 does not react with water soluble MTS reagents, the amino acids adjacent to R57 and K361 should be mutated to cysteine residues sequentially one at a time. MTS reagents will be used to probe the solvent accessibility of each mutated amino acid, and transport activity will be quantified in the absence and presence of MTS reagent to investigate substrate accessibility to the mutated amino acid. Cysteine scanning will allow us to identify important amino acids for substrate binding and /or translocation.

The results in specific aim1 suggest that OATP1B1 may have multiple sites for substrate binding. It is also possible that once the substrate is attracted to the

transporter, there are multiple domains along the translocation pathway to facilitate different substrates across the membrane. The primary study in specific aim 1 suggests that positive charge could be important in substrate binding, however, simply providing another positive charged amino acid at certain positions in transmembrane domains does not rescue the impaired translocation process. Given the amphipathic nature of OATP/Oatp substrates, aromatic amino acids such as tyrosine, tryptophan, and phenylalanine could form aromatic domains inside the binding pocket to translocate the substrates across the membrane through interactions within these aromatic groups. Therefore, to investigate the roles of conserved aromatic residues, especially those within the transmembrane domains, is another approach to study the mechanism of OATP/Oatp mediated transport. Aromatic residues will be mutated to size matched non-aromatic residues. Surface expression and transport function of the mutants will be tested.

OATP1B1 and OATP1B3 are two closely related transporters. They share only one rodent homologue Oatp1b2. Whether OATP1B1 and OATP1B3 can function as either homo-dimer or hetero-dimer is still unknown. One straight forward way to study dimerization is to establish the evidences of co-localization. DNA constructs containing either His-tagged or HA tagged OATP1B1 will be co-transfected into HEK293 cells. Cross-linking reagents with different chemistry and different chain lengths will be used to cross-link the nearest two transporters. Then, immunoprecipitation will be carried out using one epitope tag and western blot will be used to

detect the other epitope tag. If there is a positive band on the western blot, further analysis such as massspectrometry can be used to identify the reactive group of cross-linking and possibly the distance between the two proteins.

Multiple binding sites for different substrates on Oatp1a4 have also been suggested in specific aim 2. Therefore, mapping the domains that are critical for the different substrates is also important in understanding the structure-function relationship of OATP/Oatp. Thus, additional chimeras between Oatp1a1 and Oatp1a4 chimeras will be made. Each transmembrane domain will be reciprocally and sequentially exchanged. For example, the first set of chimeras has the first transmembrane domain swapped between Oatp1a1 and Oatp1a4, and the second set of mutants has the first two transmembrane domains swapped between the two transporters *etc.* Transport of different substrates will be quantified and the minimum structural requirements for transport function will be evaluated. Combined with computer modeling to compare the 3D structure differences between different chimeras, it is possible to identify critical domains for the transport of different substrates.

In specific aim 2, EL4 and TM8 have been identified to be important for the transport of several Oatp1a4 substrates. A similar strategy can be used to study the human orthologue OATP1A2. Substrate specificity will be determined among OATP1A2, Oatp1a1 and Oatp1a4 and the amino acid sequence of the three

transporters will be compared at the same time. If a compound is found to be a good substrate for two out of three transporters, then the amino acid differences between the transporters could be used to determine the substrate specificity. Site-directed mutagenesis then will be used to investigate the roles of these amino acids in substrate selectivity of OATP1A2 mediated transport.

An interesting observation was made in the surface expression experiment of chimeric proteins in specific aim 2. Chimera 3 was only partially glycosylated on the cell surface, and chimera 3 did not have transport function for any of the substrates tested. Therefore, whether glycosylation plays a role in OATP/Oatp mediated transport activity is another question to be answered. Previous research has shown that when the N-glycosylation sites in Oatp1a1 were mutated, transport activity was abolished because the transporter was not expressed at the cell membrane (Wang et al., 2008). Therefore, glycosylation is important for surface expression of Oatp1a1. To test whether glycosylation status is directly involved in transport processes, uptake of model substrates will be quantified in empty vector transfected cells and OATP/Oatp expressing cells before or after deglycosylation. In this way, the transporter is present on the surface. If there is any decreased transport activity due to deglycosylation treatment, the glycosylation state might be important for transport function.

All above mentioned strategies will lead to a better understanding of the substrate recognition and transport mechanism of the OATP/Oatp family of multispecific transporters.

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Zhou JJ, Fatehi M and Linsdell P (2008) Identification of positive charges situated at the outer mouth of the CFTR chloride channel pore. *Pflugers Arch* **457**:351-360.

APPENDICES

Appendix I: Citations of Publication

1. Gui, C., **Miao, Y.**, Thompson, L., Wahlgren, B., Mock, M., Stieger, B., and Hagenbuch, B. (2008). Effect of pregnane X receptor ligands on transport mediated by human OATP1B1 and OATP1B3. *European journal of pharmacology* **584**, 57-65.
2. Cui, Y. J., Cheng, X., **Weaver, Y. M.**, and Klaassen, C. D. (2009). Tissue distribution, gender-divergent expression, ontogeny, and chemical induction of multidrug resistance transporter genes (Mdr1a, Mdr1b, Mdr2) in mice. *Drug metabolism and disposition: the biological fate of chemicals* **37**, 203-10.
3. **Weaver, Y. M.**, Ehresman, D. J., Butenhoff, J. L., and Hagenbuch, B. (2010). Roles of rat renal organic anion transporters in transporting perfluorinated carboxylates with different chain lengths. *Toxicol Sci* **113**, 305-14.
4. **Weaver, Y. M.**, and Hagenbuch, B. The role of conserved positively charged amino acids in OATP1B1 mediated transport. *J Membr Biol* submitted.
5. **Weaver, Y. M.**, Hammann-Hänni, A., van Montfoort, J., Meier-Abt, F., Grütter, M. G., Meier, P. J., and Hagenbuch, B. The role of extracellular loop 4 and transmembrane domain 8 in Oatp1a4 mediated transport of multiple substrates. *In preparation*.
6. **Weaver, Y. M.**, Ehresman, D. J., Chang, S., Butenhoff, J. L., and Hagenbuch, B. The roles of organic anion transporters in renal elimination of branched and linear perfluorobutyrate in rats. *In preparation*

Appendix II: Citations of Peer-reviewed Presentation/Poster Abstracts

1. **Miao, Y.** and Hagenbuch B. Conserved Positively Charged Amino Acid Residues in the Putative Binding Pocket are Important for OATP1B1 Function. **Experimental Biology Meeting, April 2007**, Washington D.C. **Symposia Presentation.**
2. **Weaver, Y. M.** and Hagenbuch, B. Identification of transporters involved in renal elimination of perfluorinated carboxylates in rats. **Central State SOT Meeting, September, 2008**, University of Kansas Medical Center, Kansas City, KS. **Poster.**
3. **Weaver, Y. M.** Conserved Positively Charged Amino Acid Residues Facing the Putative Binding Pocket Are Important for OATP1B1 Function. **Biomedical Research Training Program. May 2009**, University of Kansas Medical Center, Kansas City, KS. **Presentation.**
4. **Weaver, Y. M.** and Hagenbuch, B. . Identification of transporters involved in renal elimination of perfluorinated carboxylates in rats. **SOT Annual Meeting, March 2009**, Baltimore, MD. **Poster.**
5. **Weaver, Y. M.** , Ehresman, D. J., Chang, S., Butenhoff, J. L. and Hagenbuch, B. . The roles of organic anion transporters in renal elimination of branched and linear perfluorobutyrate in rats. **SOT Annual Meeting, March 2010**, Salt Lake City, UT. **Poster.**

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